

From DEPARTMENT OF MEDICINE
Karolinska Institutet, Stockholm, Sweden

**HORMONAL INFLUENCES ON
CHOLESTEROL AND BILE ACID
METABOLISM:
FOCUSING ON INVOLVEMENT OF PCSK9**

Moumita Ghosh Laskar



**Karolinska
Institutet**

Stockholm 2018

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet.

© Moumita Ghosh Laskar, 2018

ISBN 978-91-7831-053-1

Printed by E-Print AB 2018

(<http://www.eprint.se>)

Hormonal influences on cholesterol and bile acid metabolism: Focusing on involvement of PCSK9

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Location: Hörsalen, Novum, Blickagången 6, Karolinska Institute, Huddinge

Date and time: Friday, 1st June, 2018, 09:00 am

By

Moumita Ghosh Laskar

Principal Supervisor:

Mats Rudling
Karolinska Institute
Department of Medicine, Huddinge
Division of Metabolism

Opponent:

Prof. Jan Oscarsson
Göteborg University
Department of Neuroscience and Physiology
AstraZeneca AB, Mölndal

Co-supervisors:

Bo Angelin
Karolinska Institute
Department of Medicine, Huddinge
Division of Metabolism

Examination Board:

Prof. Ewa Ehrenborg
Karolinska Institute
Department of Medicine
Division of Center for Molecular Medicine

Lena Beckman
Karolinska Institute
Department of Medicine, Huddinge
Division of Metabolism

Prof. Gunilla Olivecrona
Umeå University
Department of Medical Biosciences
Division of Physiological Chemistry

Dr. Stefan Arver
Karolinska Institute
Department of Medicine
Division of ANOVA-Andrology,
Sexual Medicine, Trans Medicine

To My Family

"Education is not the learning of facts, but the training
of the mind to think"

Albert Einstein

ABSTRACT

Elevated plasma LDL-C is today considered a causative factor for the development of atherosclerosis. Increased plasma LDL-C levels are mainly due to reduced numbers of functional LDL receptors (LDLRs) in the liver. PCSK9 and IDOL, are two, recently identified key regulators of cholesterol metabolism, both reducing LDLR numbers in the liver and peripheral tissue thereby increasing plasma LDL-C levels. Several hormones such as estrogen, testosterone and ACTH are known to influence lipoprotein metabolism. In addition, bile acids such as CDCA have been reported to modulate cholesterol metabolism. However, little is known about the underlying mechanisms behind these effects.

In this study, we investigated how natural changes of the endogenous levels of estrogen and how treatments with testosterone, ACTH, and with the natural FXR agonist (CDCA) influence cholesterol metabolism, with a particular focus on the role of PCSK9. It is concluded that:

- 1) The natural changes of estrogen levels during the menstrual cycle correlate to the concomitant changes observed in circulating levels of PCSK9 and LDL-C. Further, after menopause PCSK9 levels and plasma LDL-C increase while in ageing men of similar age PCSK9 levels were stable.
- 2) High dose testosterone treatment reduces PCSK9 levels, indicating that this LDLR modulator is under hormonal control. However, the known gender differences in bile acid and cholesterol synthesis cannot be explained from testosterone levels in human.
- 3) The adrenal glands are practically devoid of the PCSK9 protein while instead IDOL is expressed. Treatment with ACTH suppresses adrenal IDOL expression contributing to the powerful increase in LDLR number during severe stress. Simultaneously, the number of hepatic LDLRs is reduced through a posttranscriptional increase of PCSK9 protein. This effect is dependent on the presence of intact adrenals, compatible with the concept that a sustained adrenal production of corticosteroids is secured by shunting LDL-C to the adrenals in situations of severe stress.
- 4) CDCA influences lipid metabolism by reducing plasma clearance of LDL-C. The reduction of circulating PCSK9 by CDCA treatment may counter-balance its effect on hepatic LDLRs and plasma LDL-cholesterol.

LIST OF SCIENTIFIC PAPERS

- I. **Ghosh M**, Gälman C, Rudling M, Angelin B. Influence of physiological changes in endogenous estrogen on circulating PCSK9 and LDL cholesterol. J Lipid Res. 56(2):463-469 (2015)
- II. **Ghosh Laskar M**, Beckman L, Laskar A, Gårevik N, Ekström L, Rudling M, Angelin B. Testosterone reduces circulating PCSK9 but does not influence cholesterol or bile acid synthesis in healthy males. Submitted (2018)
- III. **Ghosh Laskar M**, Angelin B, Rudling M. Differential regulation of LDL receptors by ACTH: posttranscriptional control by PCSK9 in the liver and by IDOL in the adrenals. Manuscript (2018)
- IV. **Ghosh Laskar M**, Eriksson M, Rudling M, Angelin B. Treatment with the natural FXR agonist chenodeoxycholic acid reduces clearance of plasma LDL while decreasing circulating PCSK9, Lp(a), and apo C-III. J Intern Med. 281(6):575-585 (2017)

OTHER SCIENTIFIC PAPERS (NOT INCLUDED IN THE THESIS)

- I. Li W, Laskar A, Sultana N, Osman E, **Ghosh M**, Li Q, Yuan XM. .Cell death induced by 7-oxysterols via lysosomal and mitochondrial pathways is p53-dependent. Free Radic Biol Med. 53(11):2054-2061(2012)
- II. Laskar A, **Ghosh M**, Khattak SI, Li W, Yuan XM. Degradation of superparamagnetic iron oxide nanoparticle-induced ferritin by lysosomal cathepsins and related immune response. Nanomedicine (Lond). 7(5):705-717 (2012)
- III. Li W, **Ghosh M**, Eftekhari S, Yuan XM. Lipid accumulation and lysosomal pathways contribute to dysfunction and apoptosis of human endothelial cells caused by 7-oxysterols. Biochem Biophys Res Commun. 409(4):711-716 (2011)
- IV. **Ghosh M**, Carlsson F, Laskar A, Yuan XM, Li W. Lysosomal membrane permeabilization causes oxidative stress and ferritin induction in macrophages. FEBS Lett. 585(4):623-629 (2011)

TABLE OF CONTENTS

1. INTRODUCTION.....	1
1.1 Cholesterol metabolism.....	1
1.2 Lipoprotein metabolism.....	2
1.3 Bile acid metabolism.....	5
1.4 LDLR.....	7
1.5 PCSK9.....	8
1.6 IDOL.....	9
1.7 Hormonal regulation of cholesterol metabolism.....	11
2. AIMS	14
3. MATERIALS AND METHODS	15
3.1 Animals, human subjects, and study design.....	15
3.2 Serum analysis.....	16
3.3 Tissue analysis.....	17
3.4 Western blot	18
3.5 Quantitative real-time PCR.....	18
3.6 Statistics.....	19
4. RESULTS AND COMMENTS.....	20
4.1 Paper 1	20
4.2 Paper II	22
4.3 Paper III.....	23
4.4 Paper IV.....	24
5. DISCUSSION AND FUTURE PROSPECTIVE.....	26
5.1 Estrogen and PCSK9.....	26
5.2 Testosterone and PCSK9	27
5.3 Testosterone and gender dependent cholesterol and bile acid synthesis	27
5.4 ACTH and PCSK9	28
5.6 CDCA, lipoproteins and PCSK9	29
5.7 Relevance of PCSK9 regulation	29
6. CONCLUSIONS	31
7. ACKNOWLEDGEMENTS.....	32
8. REFERENCES	34

LIST OF ABBREVIATIONS

ACTH	Adenocorticotrophic hormone
ADX	Adrenalectomized
Apo	Apolipoprotein
BAAT	Bile acid coenzyme A:amino acid N-acyltransferase
BSEP	Bile salt export pump
CA	Cholic acid
C4	7 α -hydroxy-4-cholesten-3-one
CDCA	Chenodeoxycholic acid
CETP	Cholesteryl ester transfer protein
CVD	Cardiovascular disease
CYP7A1	Cholesterol 7 α -hydroxylase
DCA	Deoxycholic acid
E2	β -estradiol
ER	Estrogen receptor
FC	Free cholesterol
FGF	Fibroblast growth factor
FGFR4	Fibroblast growth factor receptor 4
FPLC	Fast performance liquid chromatography
FXR	Farnesoid X receptor
GC-MS	Gas chromatography-mass spectrometry
GH	Growth hormone
GOF	Gain of function
HDL	High-density lipoprotein
HL	Hepatic lipase
HMGCoAR	3-hydroxy-3-methylglutaryl coenzyme A reductase
IBAT	Ileal bile acid transporter
IDL	Intermediate density lipoprotein
IDOL	Inducible degrader of LDL receptor
INSIG	Insulin induced gene
LCA	Lithocholic acid
LCAT	Lecitin:cholesterol acyltransferase
LC-MS-MS	Liquid chromatography-mass spectrometry-mass spectrometry
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LDLR	Low density lipoprotein receptor
LOF	Loss of function
Lp(a)	Lipoprotein(a)
LPL	Lipoprotein lipase
LRP	Low density lipoprotein receptor-related protein
LRH-1	Liver receptor homolog-1
LXR	Liver X receptor
MTTP	Microsomal triglyceride transfer protein
MYLIP	Myosin regulatory light chain interacting protein
NPC1L1	Niemann-Pick C1 Like 1
NTCP	sodium bile acid co-transporter
OST	Organic solute transporter
PCR	Polymerase chain reaction
PCSK9	Proprotein Convertase Subtilisin Kexin Type 9
PL	Phospholipids
SHP	Small heterodimer
SREBP	Sterol regulatory element binding protein
TG	Triglycerides
VLDL-C	Very low density lipoprotein cholesterol

1. INTRODUCTION

In humans, an increased risk for development of atherosclerosis and cardiovascular events is associated with disturbances in cholesterol metabolism. Epidemiological studies have demonstrated that LDL cholesterol (LDL-C), age, gender, blood pressure, diabetes, smoking, obesity, family history, and an unhealthy diet are important risk factors for atherosclerosis. In humans, LDL cholesterol is now considered a causative factor for the development of arteriosclerosis. (1, 2).

Cholesterol metabolism can be altered by several factors. Hormones such as estrogen, thyroid hormone (TH), adrenocorticotrophic hormone (ACTH), and growth hormone (GH) have been reported to influence cholesterol homeostasis (3-8). Proteins such as the low-density lipoprotein receptor (LDLR), sterol regulatory element-binding protein 2 (SREBP2), proprotein convertase subtilisin/kexin type 9 (PCSK9) and apolipoprotein B (ApoB) play crucial roles in cholesterol metabolism (9). Moreover, environmental factors such as diet and life style are important for cholesterol metabolism.

1.1 Cholesterol metabolism

Cholesterol is of fundamental importance for normal cell function and serves as a precursor in the synthesis of bile acids and of steroid hormones such as estrogens, testosterone, and the glucocorticoids. Cells acquire cholesterol by de novo synthesis (endogenous pathway) and from the diet (exogenous pathway).

Endogenous pathway: Although all nucleated cells can produce cholesterol, the liver and intestine play the primary role in cholesterol synthesis in humans and animals. 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) is the rate-limiting enzyme in cholesterol synthesis. HMGCoAR is an integral membrane protein, which is regulated via negative feedback mechanism through the SREBP pathway. When intracellular cholesterol is low, SREBP2 is activated by cleavage and translocated to the nucleus where it initiates the transcription of HMGCoAR (1, 2). When cholesterol is in excess within cells, SREBP2 is inactivated which negatively regulates cholesterol synthesis (10, 11). Cholesterol synthesis has a diurnal rhythm that peaks at midnight (10). Statins, the most commonly used lipid-lowering drugs bind strongly to the HMGCoAR protein thereby inactivating the enzyme activity. (12).

Exogenous pathway: After food intake, dietary lipids are hydrolyzed by pancreatic lipases and form micelles with bile acids that are absorbed by brush border membrane of enterocytes

(13). Cholesterol enters the brush border membranes either via different transporters such as Niemann-Pick C1 Like 1 (NPC1L1) (14), or by passive diffusion, and is then re-esterified by acetyl-Coenzyme A acetyltransferase 2 (ACAT2) (15). Whereas ATP-binding cassette transporters G5 and G8 (ABCG5/8) preferentially pump out plant sterols, they also help to transport back absorbed cholesterol into the intestinal lumen (16-18). Within the enterocyte, esterified sterol assembles with triglycerides (TGs) and apolipoproteins in the presence of microsomal triglyceride transfer protein (MTTP) and promotes lipoprotein formation. Lipoproteins such as Chylomicrons and VLDL reach the circulation from the enterocyte via the lymphatic system (**Fig 1**) (19, 20).

1.2 Lipoprotein metabolism

Lipoproteins are classified into five classes: chylomicrons, very low-density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (21-27) (**Table 1**). High amounts of plasma cholesterol, especially LDL-C and VLDL-cholesterol (VLDL-C), as well as low levels of HDL-cholesterol (HDL-C) are linked to CVD. Moreover, subclasses of lipoproteins such as Lipoprotein (a) (Lp(a)) may also be atherogenic.

Chylomicrons

Chylomicrons are the largest lipoproteins and are formed postprandially in the enterocytes. They deliver FFAs to peripheral tissues in a similar way as VLDL particles are then removed by LDLRs or by LDLR-related proteins (LRP) in the liver (27).

VLDL

Nascent VLDL particles, are secreted from the liver and contain ApoB100, apolipoprotein C1 (ApoC1) and apolipoprotein E (ApoE). In the circulation, they pick up apolipoprotein C-II (ApoC-II) and additional apoE, forming mature VLDL. These particles are then hydrolyzed by LPL to release free fatty acids (FFA) which are taken up by peripheral tissues. Thereafter, VLDL remnant (IDL) particles are either cleared by LDLRs or metabolized into LDL by hepatic lipase (HL) (25, 26).

IDL

IDL is the intermediate density lipoprotein between LDL and VLDL. IDL is produced from the degradation of the larger, triglyceride-rich VLDL. However, it is quickly cleared from

circulation. Either hepatic LDLRs remove the IDL particle from the circulation or it is converted to LDL moieties (24).

LDL

LDL particles are formed from IDL and deliver cholesterol to cells by LDLR-mediated endocytosis. LDLs can also be taken up by macrophages and initiate the atherogenic process. To reduce the risk of CVD by lowering LDL-C is a primary target of lipid lowering therapy (23).

Table 1: Major lipoproteins and their characteristics.

Lipoproteins	Density (g/mL)	Diameter (nm)	TG %	Chol %	PL %	Protein %	Half- life	Surface Apolipoproteins
Chylomicron	0.93	75-1200	80-95	2-5	3-8	1-2	15 min	ApoB48, ApoAI, ApoAII, ApoAIV, ApoCII and ApoE.
VLDL	0.93-1.006	30-80	50	22	19	8	2 h	ApoB100, ApoC, ApoE
IDL	1.006-1.019	25-35	20	38	23	19	2-6 h	ApoB100 and ApoE
LDL	1.019-1.063	18-25	11	47	22	21	2-3 d	ApoB100
HDL	1.063-1.21	5-12	6	15-22	23-30	55	2-3 d	ApoAI, ApoAII, ApoAIV, ApoC ApoE

HDL

An important function of HDL is to transport cholesterol from peripheral tissues to liver a pathway designated reverse cholesterol transport. HDL removes excess cholesterol during the process called "HDL biogenesis" which occurs through a complex pathway that involves the lipid transporter ATP-binding cassette A1 (ABCA1) and the plasma enzyme lecithin cholesterol acyl transferase (LCAT). Although a high plasma level of HDL seems to protect

against cardiovascular events, clinical trials where HDL has been pharmacologically increased have failed to reduce such events (21, 22).

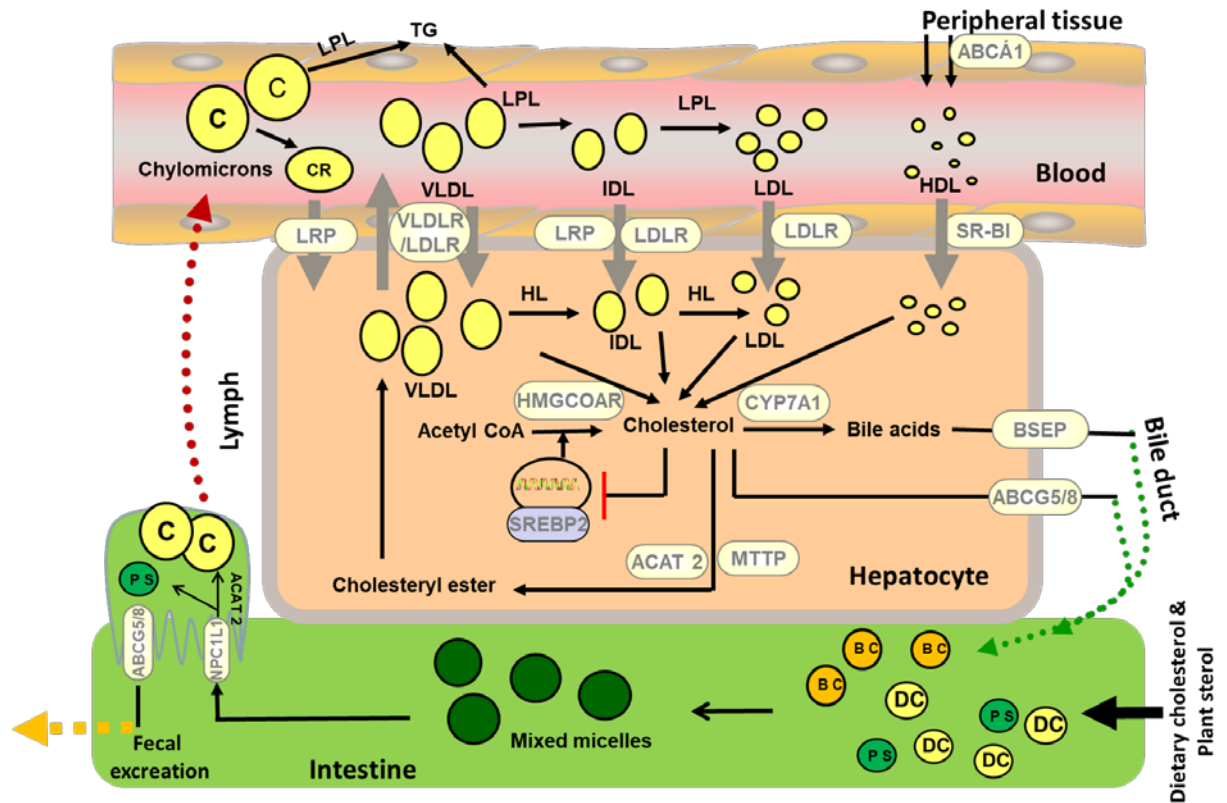


Fig1: Schematic diagram of cholesterol and lipoprotein metabolism.

Dietary cholesterol (DC); Biliary cholesterol (BC); Plant sterol (PS)

Lp(a)

Lp(a) is an LDL particle with an apo(a) molecule covalently bound to ApoB. The level of Lp(a) is highly influenced by polymorphisms of the Apo(a) gene and is linked to the risk for CVD. Since Apo(a) is expressed in liver cells, Lp(a) is believed to be synthesized in the hepatocyte where Apo(a) assembles with LDL. LDLRs may partially be involved in the plasma clearance of Lp(a), but the metabolism of Lp(a) is incompletely understood. The half-life of Lp(a) is 3-4 days which is longer than LDL. The kidneys are also considered to play an important role in Lp(a) clearance from plasma (28-31).

Apolipoproteins

Apolipoproteins are the protein part of lipoproteins, synthesized in liver or intestine. Apolipoproteins together with phospholipid and free cholesterol surround the lipid particles. Apolipoproteins work as ligands for various receptors or as activators of enzymes, influencing the cellular uptake of lipoprotein particles. Apolipoproteins are mainly classified into six groups with several sub-groups. These are Apo A (Apo AI, Apo AII, Apo AIV and Apo AV), Apo B (Apo B48 and Apo B100), Apo C (Apo CI, Apo CII, Apo CIII and Apo CIV), Apo D, Apo E, Apo H and Apo L.

1.3 Bile acid metabolism

Bile acids are derivatives of cholesterol produced in the liver. Every day, about 500mg cholesterol, is converted to bile acids. Previously bile acids were mainly considered as detergents, facilitating lipid absorption, but studies have established several other important functions.

Primary bile acids

Bile acids are synthesized through a "classical" and an "alternative" pathways. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the two primary bile acids in humans, and 90% of bile acids are formed by the classical pathway. Cholesterol 7 α -hydroxylase (CYP7A1), a member of the cytochrome P450 superfamily, is the rate-limiting enzyme for the classic pathway. Cholesterol is hydroxylated by CYP7A1 and then metabolized to 7 α -hydroxy-4-cholesten-3-one (C4) by 3 β -hydroxysteroid hydrogenase (HSD3B7). C4 serves as a precursor for CA and CDCA. Therefore, serum C4 levels can be used as a marker for bile acid synthesis (32, 33) (**Fig 2**).

Secondary bile acids

About 5% of bile acids that enter the gut escape absorption in the small intestine, to enter the large intestine where they are dehydroxylated into secondary bile acids by bacteria. In humans and rodents, deoxycholic acid (DCA), lithocholic acid (LCA) and small amounts of ursodeoxycholic acid (UDCA) are produced as secondary bile acids. These secondary bile acids are then either passively absorbed and resecreted or excreted in feces (34, 35).

Enterohepatic circulation

Bile acids circulate in the enterohepatic system several times after a meal. After food intake, the gallbladder contracts and empties bile into the gut where they contribute to micelle

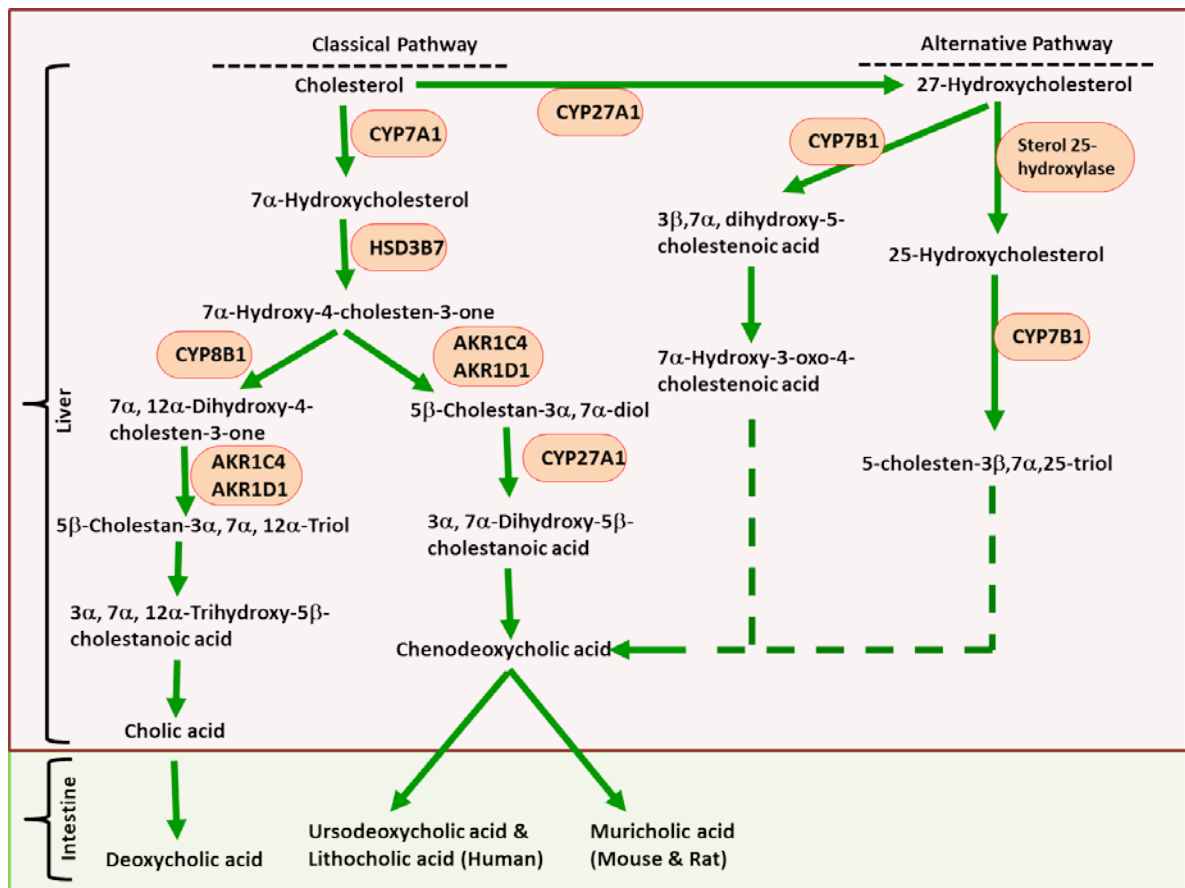


Fig 2: Bile acid synthesis (classical and alternative pathway)

(AKR1C4: 3 α -hydroxy steroid dehydrogenase

AKR1D1: Oxosteroid-5 β -reductase

CYP7A1: Cholesterol 7 α -hydroxylase

CYP8B1: Sterol-12 α -hydroxylase

CYP27A1: Sterol 27-hydroxylase

HSD3B7: 3 β -hydroxy steroid dehydrogenase)

formation and fat absorption. In the distal ileum, ASBT mediates the absorption of bile acids (about 95%) by the enterocytes. Thereafter bile acids are exported via organic solute transporters (OSTs) into the blood and transported back to the liver in the portal vein (36, 37). The hepatocytes extract bile acids from portal blood, driven by sodium-taurocholate co-transporting polypeptide (NTCP). In the liver, bile acids bind to Farnesoid X receptors (FXR) and suppress bile acid synthesis by down-regulating CYP7A1 (**Fig 3**). Primary bile acids such as CDCA and CA are natural FXR agonists that suppress bile acid synthesis by inhibiting CYP7A1 (38, 39). Another mechanism for feedback regulation of bile acid synthesis involves fibroblast growth factor 19 (FGF19) [fibroblast growth factor 15 (FGF15) in rodents]. This protein is secreted from enterocytes and transported to the liver where it binds to the FGF receptor 4 (FGFR4) which then down-regulates the expression of CYP7A1,

resulting in suppression of bile acid synthesis (40). Although FGF19 is detectable in the human circulation, the presence of plasma FGF15 in the rodent is controversial. However, a recent study has claimed that FGF15 is present in mouse serum (41).

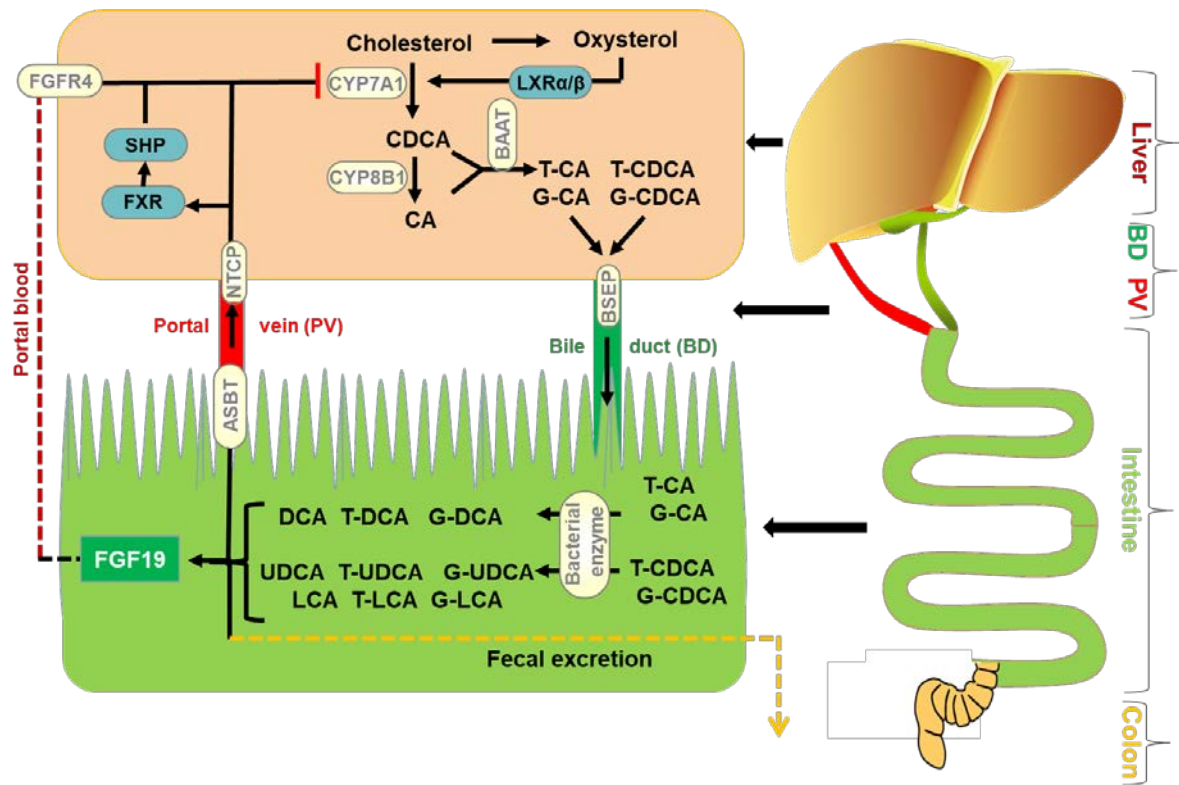


Fig 3: Schematic diagram of bile acid metabolism

1.4 LDLR

LDLRs are found in all mammalian cells but at highest density in the adrenal gland. The liver expresses a major part of all LDLRs (42, 43). The LDLR is a five-domain membrane-bound glycoprotein, which is synthesized in the endoplasmic reticulum and transported to the cell membrane via the Golgi complex. LDLRs bind to both ApoB100 and ApoE, the affinity being higher for ApoE (44). LDLRs bind and internalize LDL particles and promote their lysosomal degradation (45, 46). Mutations in the LDLR gene, located on chromosome 19, are the most frequent cause of familial hypercholesterolemia (FH) (47, 48).

LDLR is transcriptionally regulated by SREBP2 (49). However, several recent studies have established the importance of post-transcriptional regulation of LDLR. Proprotein convertase

subtilisin/kexin type 9 (PCSK9) and Inducible Degradator of the LDL receptor (IDOL) are two such molecules, which can modulate the degradation of LDLRs (50).

1.5 PCSK9

PCSK9, the ninth member of the proprotein convertase family, is also known as neural apoptosis-regulated convertase 1. It was discovered in 2003 (51, 52). PCSK9 is abundant in the liver, and also found in the small intestine, kidney, and the central nervous system. In humans, the PCSK9 gene is located on chromosome 1p32.3 (53). Variants of this gene result in abnormal cholesterol metabolism. Gain of function (GOF) mutations lead to hypercholesterolemia, whereas loss of function (LOF) variants cause hypocholesterolemia and reduced risk of CVD (54). Several studies have established clear correlations between PCSK9 and cardiovascular complications (51-53).

PCSK9 is secreted from the liver. Its precursor (72 Kda) is synthesized in the hepatocyte and produces a pro-segment and mature PCSK9 (63 KDa) via autocatalytic cleavage (55). Mature PCSK9 undergoes trafficking from ER to Golgi before secretion into the circulation (54, 55). Some post-translational modifications such as N-glycosylation, sulphation, and phosphorylation can take place before its secretion (53). The catalytic domain of circulating PCSK9 binds with the epidermal growth factor precursor homology domain-A (EGF-A) of the LDLR to form a LDLR/PCSK9 complex (56). This complex is then internalized via clathrin-mediated endocytosis (57) and is directed to the lysosome for degradation (57-60) (**Fig 4**). Recent studies have shown that intracellular PCSK9 can also promote LDLR degradation by binding to the LDLRs inside the cells (**Fig 4**) (54, 61).

Regulators of PCSK9

Several physiological and pharmacological parameters regulate the PCSK9 level and function. Physiological parameters such as age, gender and hormones have been proposed to be important in the regulation of PCSK9. Higher levels of circulating PCSK9 are found in females than in males. While pre-menopausal females have lower PCSK9 level than males, the level increases after menopause. In males, the level is quite stable throughout life (62, 63). Moreover, it has been reported that various parameters such as LDL-C, total-C, triglycerides, insulin, body mass index (BMI), and glucose are positively correlated with plasma PCSK9 levels. Hormones such as estrogen, thyroid hormone, and glucagon have been reported to regulate PCSK9 as well. Diet has also been shown to be important for regulation of PCSK9 in animals and humans. Sterols affect PCSK9 mRNA expression via SREBP2 pathway (64, 65). Mediterranean diet and food rich in polyunsaturated fatty acids (PUFAs)

reduce circulating PCSK9 in humans (66-68). Moreover, PCSK9 follows a diurnal rhythm in humans (69, 70).

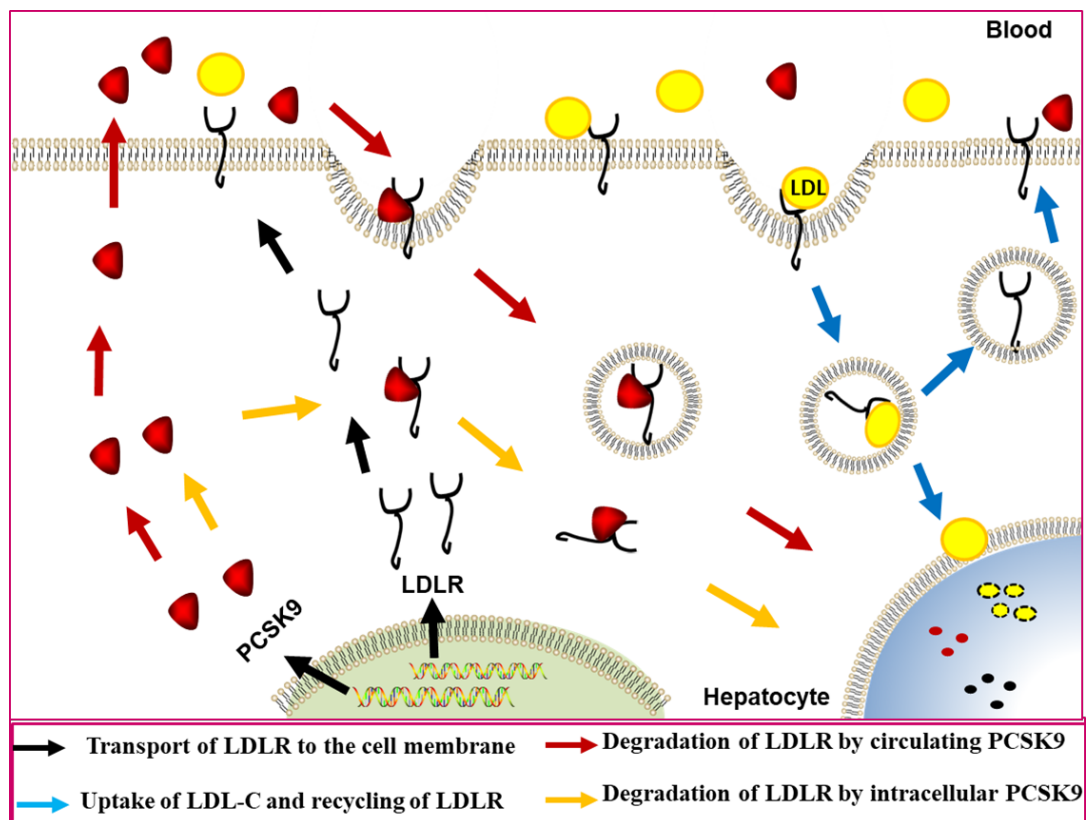


Fig 4: PCSK9 (intracellular and circulating) inhibits cellular uptake of LDL-C by degrading LDLR

PCSK9 as a therapeutic target

A decade of research has established that lowering of circulating and intracellular PCSK9 is a promising therapeutic approach for the treatment of dyslipidemia (53, 55, 62). Additionally, the commonly used lipid-lowering drugs such as statins and ezetimibe induce PCSK9 in humans to dampen their therapeutic effects. Inhibition of PCSK9 either by monoclonal antibodies or by molecules such as siRNA, peptides and small molecules are now undergoing clinical trials in different phases (71).

1.6 IDOL

IDOL, also known as Myosin regulatory light chain interacting protein (MYLIP), is a recently identified protein reported to regulate cholesterol homeostasis independent of SREBP pathway. Studies have demonstrated that loss-of-function mutations of the IDOL

gene lower plasma cholesterol levels (72), whereas polymorphisms in the gene region are associated with elevated plasma cholesterol in humans (73).

IDOL and LDLR

Liver X receptor (LXR) is a nuclear receptor which regulates IDOL transcriptionally. Upon activation, LXR/retinoid X receptor (RXR) heterodimers bind to the promoter region of IDOL gene and regulate its function (74). Oxysterols work as ligands for LXRs (LXR α and LXR β) and activate them (75). LXR activation up-regulates IDOL expression in the cells independent of clathrin-mediated endocytosis which then ubiquitinates LDLR by E3 ubiquitin ligase and causes multi-vesicular body-mediated lysosomal degradation of the LDLR (**Fig 5**) (74-77).

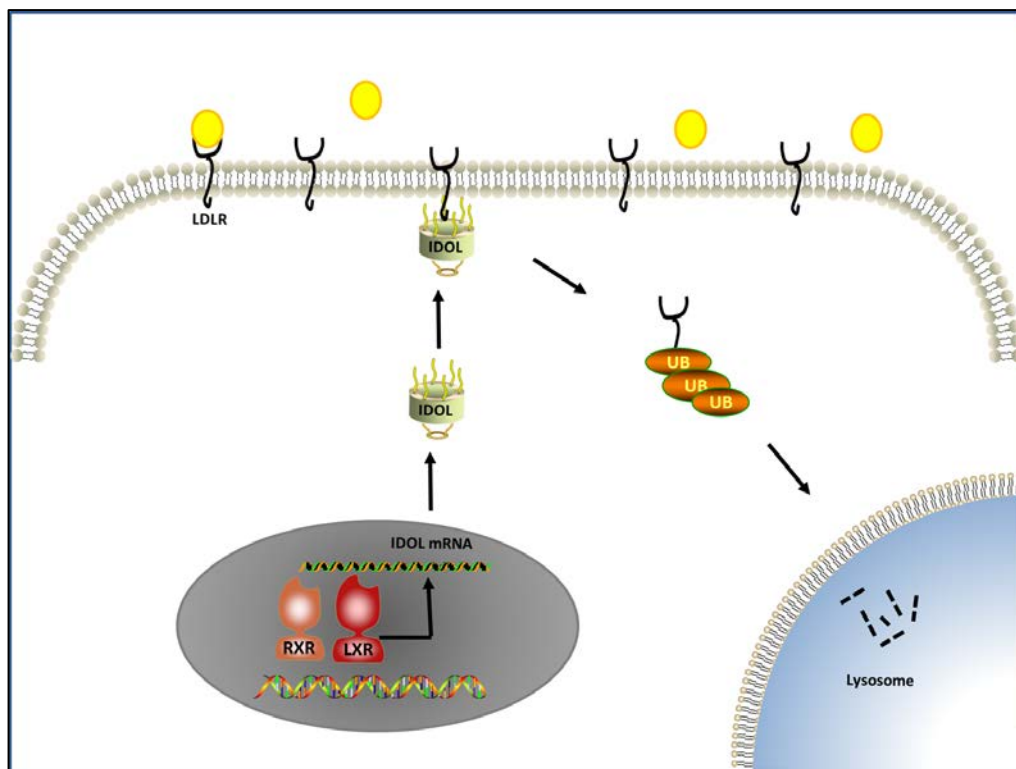


Fig 5: Degradation of LDLR by IDOL

IDOL is found in various organs such as liver, intestine, spleen and adrenal gland in humans (76, 82, 83). However, studies have resolved IDOL as tissue and species-specific gene (84). It has been shown that LXR agonists enhance IDOL expression in peripheral tissue but not

in the liver in mice, whereas in cynomolgus monkeys, LXR agonist induces IDOL expression in liver and increases plasma LDL-C (84).

Additionally, IDOL may ubiquitinate and promote the degradation of other LDLR family members, such as very low-density lipoprotein receptor (VLDLR) and ApoE receptor 2 (ApoER2) (78, 79).

1.7 Hormonal regulation of cholesterol metabolism

Hormones are signaling molecules mainly secreted by endocrine glands (**Fig 6**). A large number of hormones may regulate cholesterol and bile metabolism. In this study, we focused on three hormones, estrogen, testosterone, and ACTH, which have previously been reported to influence cholesterol metabolism.

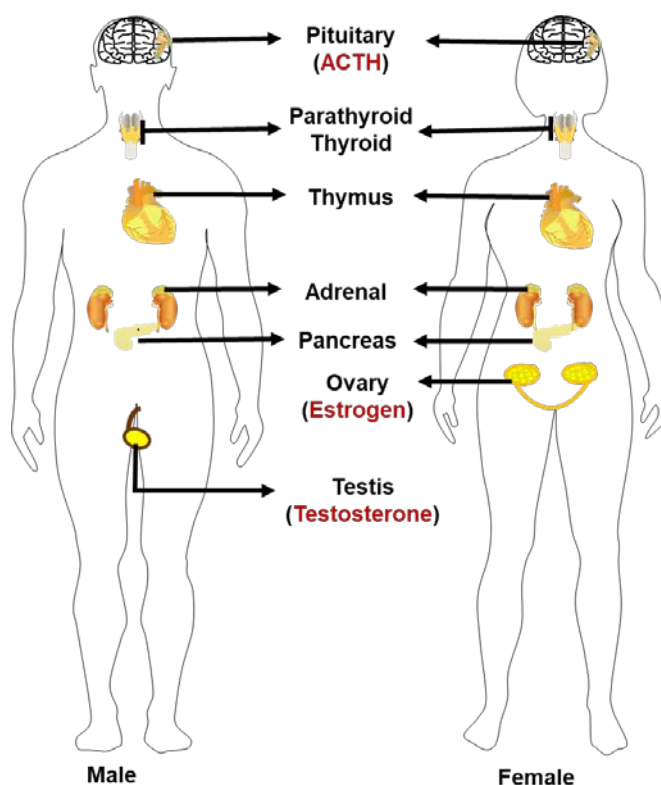


Fig 6: Hormone producing glands in human (male and females)

Estrogen

Estrogen is a female sex hormone and important for reproduction. In females, estrogen is produced by the ovaries upon stimulation by luteinizing hormone (LH) and follicle

stimulating hormone FSH). Three main natural estrogens are found in females, estrone (E1), estradiol (E2), and estriol (E3). E2 is the most important sex hormone in women and is predominant during fertile years. The plasma level of E3 increases following menopause. Estetrol (E4), the fourth type of estrogen is only produced during pregnancy. Estrogen has a key role in the menstruation cycle. The level of estrogen reaches its peak at the ovulation phase and is then reduced during the follicular phase and luteal phase. Estrogen binds to estrogen receptors (ERs) located in the nucleus and at the cell membrane, and regulate various cellular functions (80).

It has been reported that elevated estrogen levels reduce LDL-C, PCSK9, and Lp(a) (6, 81) while increasing HDL-C and ApoA1 (82). In addition, estrogen influences GH pathway. Moreover, circulating LDL-C and PCSK9 are lower in pre-menopausal women and increase after menopause (63), presumably due to the decrease in estrogen level. However, whether changes in the endogenous estrogen levels such as during the menstrual cycle influence LDL-C and PCSK9 in females is not clear.

Testosterone

Testosterone is the primary male sex hormone, which maintains the reproduction function in males. Low levels of testosterone are found in females with certain diseases such as polycystic ovarian disease (PCOD) (83). Studies have claimed that testosterone may regulate lipoproteins (84). However, little is known about the effects of testosterone on cholesterol and bile acid metabolism. One study has shown that testosterone treatment reduces LDL-C by reducing PCSK9 in pigs (85). In contrast, no notable correlation was found between testosterone and PCSK9 in healthy males (86). Whether pharmacological doses of testosterone regulate cholesterol and bile acid metabolism is yet to be clear.

ACTH

ACTH is produced and secreted by the anterior pituitary gland (87). It is an important component of the hypothalamic-pituitary-adrenal axis and produced in response to stress (88). ACTH acts as a ligand for ACTH receptors that are predominantly found in the adrenal cortex. ACTH induces secretion of glucocorticoid, mineralocorticoid and androgenic steroids from the adrenal cortex, a cellular process called steroidogenesis. During stress, ACTH up-regulates adrenal lipoprotein uptake by inducing cellular receptors such as LDLR and SRB1. Cholesterol within the lipoproteins thus serves as the precursor for steroid production.

Studies in rodents have previously shown that elevated ACTH levels increase plasma cholesterol by reducing hepatic LDLR numbers without changing the mRNA levels (3), indicating post-transcriptional regulation of LDLR upon ACTH exposure. It has been demonstrated that these effects are abolished in LDLR^{-/-} mice (3), clarifying the importance of the LDLR for the development of hypercholesterolemia. However, whether PCSK9 or IDOL may post-transcriptionally regulate LDLR during prolonged stress is unknown.

2. AIMS

Specific aims for papers 1-4 were:

- I. To investigate whether physiological changes in estrogen levels during the menstrual cycle and the menopause influence circulating PCSK9 and LDL-C.
- II. To examine the effects of testosterone treatment of healthy males on circulating PCSK9 and on bile acid and cholesterol metabolism.
- III. To study the possible underlying mechanisms behind post-transcriptional regulation of hepatic LDLRs during ACTH treatment.
- IV. To examine the effects of CDCA, a natural FXR agonist, on lipoprotein metabolism.

3. MATERIALS AND METHODS

A general description of animals, human subjects, different study designs and methods used in this thesis are described below. Detailed information about each study is present in the individual papers. All participants in human studies had given informed written consent to participate in the studies, which had been approved by the Ethics Committee of Karolinska Institutet and Karolinska institutional Animal Care and Use Committee approved the animal experiments.

3.1 Animals, human subjects, and study design

Paper 1: Serum samples of 395 subjects (206 females and 189 males) were available for analysis from a previously described cohort of 435 healthy volunteers (89). Subjects had been excluded if they had a previous history of CHD, type 2 diabetes or dyslipidemia. Females taking hormonal therapy, or contraceptive pills were also excluded from the study. Overnight fasting blood samples were collected. Serum was prepared by centrifugation. Serum samples were stored at -80°C.

Paper 2: From a previously described study (90), plasma samples from 25 healthy male volunteers who were given 250mg (dose 1) or 500mg (dose 2) of intramuscular testosterone enanthate (Testoviron® Depot) were available for analysis. Fasting blood samples were collected prior to testosterone administration (Day 0), and 4 (Day 4) and 14 days (Day 14) after testosterone administration.

Paper 3: Two sets of animal experiment were performed. In the first, 36 male Sprague Dawley rats (B&K Universal AB, Sollentuna, Sweden) were used. Animals were injected subcutaneously with Tetracosactide (ACTH) (Synacthen depot, Ciba-Geigy, Horsham, Sussex, UK), or 0.9% NaCl (controls) at 0900 h and 1600 h for different time points (3 hours to 92 hours). In second set of experiments, 16 male wild-type (B6129SF2/J) and 16 PCSK9^{-/-} (B6;129S6-Pcsk9^{tm1Jdh}/J) mice were used. All mice were from Jackson Laboratory (Bar Harbor, ME, USA). Mice were treated subcutaneously with either Tetracosactide (Synacthen depot, Ciba-Geigy, Horsham, Sussex, UK), or 0.9% NaCl twice a day for 75h. At the end of the experiments, animals were anesthetized, bled by cardiac puncture, and killed by cervical dislocation. Serum was separated and stored at -80°C. Livers and adrenal glands were collected in liquid nitrogen and stored at -80°C.

Paper 4: Two human studies were performed

In experiment 1, autologous ^{125}I -LDL was injected in 12 males, and clearance of LDL from blood plasma was characterized during acute and long-term treatment with CDCA. Blood (~100 ml) was drawn in the fasting state for preparation of LDL and subsequent iodination with ^{125}I . ^{125}I -LDL was injected within 5 days of labeling, and repeated blood samples were drawn for 2-3 weeks. When the ^{125}I -radioactivity curve was in the log-linear phase, about 10-14 days after injection, CDCA administration was initiated. In 5 patients, the acute effect (6-7 days) of CDCA administration was studied. After short and long-term treatment, the fractional catabolic rate (FCR) of ^{125}I -LDL was calculated both from plasma curve and from plasma/urine ratio.

In experiment 2, 7 gallstone patients scheduled for surgery were studied. Serum was collected before and after 3 weeks of CDCA treatment. 15 untreated gallstone patients served as controls. A liver biopsy was taken during surgery.

3.2 Serum analysis

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA for human serum: Commercially available ELISA kits were used to measure PCSK9 (Circulex; CycLex, Japan), insulin (Ultra-sensitive human insulin ELISA kits, Marcodia, Sweden), FGF19 (Quantikine, R&D Systems, Minneapolis, MN, USA), β -estradiol (Demeditec diagnostics GmbH, Germany), follicle stimulating hormone (Demeditec diagnostics GmbH, Germany). All the ELISA kits were used according to manufacturer's instruction.

ELISA for animal serum: Rat serum PCSK9 was assayed by mouse/rat PCSK9 ELISA kit from Circulex, Japan and mice serum PCSK9 was analyzed by mouse PCSK9 immunoassay kit from R&D Systems Inc., Minneapolis, USA as described by manufacturers.

Serum cholesterol, Triglyceride and glucose

For paper 1 and 4, total and HDL-C, TG, and glucose were determined using standard clinical chemistry techniques. LDL-C was assessed according to Friedewald et al (91).

For paper 2 and 3, total cholesterol (Total-C) and total TG (Total-TG) were assayed with a commercial kit (Roche Diagnostics GmbH, Mannheim, Germany). Cholesterol and TG serum lipoprotein (VLDL, LDL and HDL) profiles were analyzed by fast performance liquid chromatography (FPLC) (89).

Bile acid synthesis

Serum concentration of 7 α -Hydroxy-4-cholesten-3-one (C4), a marker of bile acid synthesis, was determined by high-performance liquid chromatography (HPLC) using 7 β -hydroxy-4-cholesten-3-one as internal standard. The values were normalized for total cholesterol (C4/c) (32).

Cholesterol synthesis

Unesterified lathosterol, a marker of cholesterol synthesis, was assayed by isotope dilution mass spectrometry after the addition of deuterium-labeled internal standard as described previously. Lathosterol levels were corrected for total cholesterol (lathosterol/c) (89).

Bile acids in serum

Serum bile acids (BA) including total bile acid (TBA), unconjugated and conjugated cholic acid (CA), Deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA) and lithocholic acid (LCA) were determined by liquid chromatography tandem mass spectrometry using deuterium-labelled BA standards (92).

Serum apolipoproteins and Lipoprotein(a) (Lp(a))

Serum apolipoproteins such as apolipoprotein A1 (apoAI) (Ref No. KAI-002), apolipoprotein A11 (apoAII) (Ref No. KAI-003), apolipoprotein B (apoB) (Ref No. KAI-004), apolipoprotein CII (apoCII) (Ref No. KAI-005), apolipoprotein CIII (apoCIII) (Ref No. KAI-006) and apolipoprotein E (apoE) (Ref No. KAI-007) were analyzed according to manufacturer's instruction by using immunoturbidimetric assay kits from Kamiya Biomedical Company, Seattle, USA (93). TruLab Lp(a) kit (Ref no. 598309910046; Diasys, Germany) was used to measured serum Lp(a) (93). All the kits were analyzed by Resposn ® 910 analyzer from Diasys, Germany.

3.3 Tissue analysis

Liver TG and cholesterol

Liver lipid was extracted as described by Folch et al. TG content in liver homogenates was detected by colorimetric analysis using a commercial kit from Roche Diagnostics GmbH, Mannheim, Germany. Liver cholesterol contents were determined by gas chromatography-mass spectroscopy (GC-MS) (94).

H&E staining

Small pieces of livers were saved in 4% PFA overnight and then in 70% ethanol until the preparation of paraffin-embedded block. Tissue sections were prepared and stained with Hematoxylin and eosin (H&E) stain for histology.

Activity assay and LDLR binding assay

Cholesterol 7 α -hydroxylase (CYP7A1) and 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoAR) activities in isolated microsomal fractions were analyzed as described (95, 96). Specific heparin-sensitive LDLR binding was determined in liver homogenates as described (43, 97).

3.4 Western blot

Membrane proteins were prepared as described (6) and added to Laemmli sample buffer (Bio-Rad Laboratories) together with 50mM dithiothreitol (DDT) and then separated on 8% SDS-PAGE gels. The proteins were electrotransferred to nitrocellulose. The membranes were blocked with 5% nonfat dried milk, dissolved in TBST with 1% tween 20. The membranes were then incubated with corresponding primary antibodies (1:1000 dilution) overnight. After wash, membranes were incubated with HRP-conjugated secondary antibodies for 1h and washed and then analyzed by CCD camera and quantified by a LAS 1000 plus imager and Multi Gauge software Science Lab 2005 version 3.1 (Fuji Photo). The quantified bands are expressed as arbitrary units. LDLR polyclonal antibody (ab30532) was from Abcam and PCSK9 polyclonal antibody (10007185) from Cayman Chemicals (Ann Arbor, MI) were used for rat samples, and anti-PCSK9 antibody (ab125251) from Abcam was used for mouse samples. IDOL polyclonal antibody (LS-C119933) was from Lifespan biosciences, Inc. Anti-actin polyclonal antibody (ab8227) was used as loading control. Goat-anti-rabbit HRP conjugated secondary antibody (1858415) was from Pierce.

3.5 Quantitative real-time PCR

Total RNA was extracted from frozen liver samples using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using random-hexamer priming and Omniscript (QIAGEN, Valencia, CA). Quantitative real-time PCR was performed in an ABI Prism 7700 sequence detection system (ABI, Foster City, CA) using SYBR Green. 18S, LDLR, PCSK9, IDOL, SREBP-2 and HMG COAR genes expression were examined by using primers as mention in paper 3.

3.6 Statistics

The differences within multiple groups was compared by one-way ANOVA following Tukey's post hoc comparisons test and differences between two groups were compared by paired or non-paired student t-test. GraphPad Prism version 5 (GraphPad Software, SanDiego, CA) was used for all the comparisons. (Details are referred to in individual papers).

4. RESULTS AND COMMENTS

4.1 Paper 1

Physiological changes in endogenous estrogen levels in females are linked to serum PCSK9 levels and LDL-C

We aimed to evaluate whether the natural changes of endogenous E2 that occur during aging as well as during the different phases of the menstrual cycle are related to changes in the circulating levels of PCSK9 and LDL-C. In this study, we demonstrate the possible underlying mechanism behind the variation in LDL-C levels during different phases of menstruation cycle and show a possible reason for elevated levels of plasma PCSK9 in post-menopausal women.

In a Swedish cohort, PCSK9 levels ranged between 113 and 831 ng/mL, with a mean concentration of 290ng/ml. Mean PCSK9 levels in males (275 ng/mL) was lower than in females (304 ng/mL; $P < 0.05$). In females, plasma PCSK9 increased in parallel with LDL-C with increasing age, which was not observed in males. In males, there was no significant change of PCSK9 levels with increasing age, although plasma cholesterol increased similar to what was seen in females.

Impact of menopausal status on plasma PCSK9 level in females

To better understand the relation between age and PCSK9 level in females, we divided them into two groups; pre-menopausal (< 50 years) and post-menopausal women (> 50 years). We considered 50 years as the average age for menopause in Swedish healthy female cohort (19). In agreement with previous studies, our results also show that plasma PCSK9 in postmenopausal women (333ng/mL) was significantly higher compared to pre-menopausal women (272 ng/mL). In males, PCSK9 levels do not change with age. These results indicate a possible role of estrogen for plasma PCSK9 in females.

PCSK9 and menstrual cycle

To further understand the distribution of PCSK9 and cholesterol during menstruation cycle and their relation with E2, we sorted out pre-menopausal females according to their menstruation phases based on their reported last menstruation date. They were divided into three phases, follicular phase (days 1-11), ovulation phase (days 12-18) and luteal phase (days 13-32) (20). The E2 levels were significantly lower in the follicular phase and highest in those in the ovulation phase whereas, an average level of Serum E2 found in their luteal

phase. We found a noticeable reduction of PCSK9 in the ovulation phase and in the luteal phase as compared to the follicular phase. Interestingly, LDL-C and total-C followed the same trend as PCSK9 (Fig 7A-D).

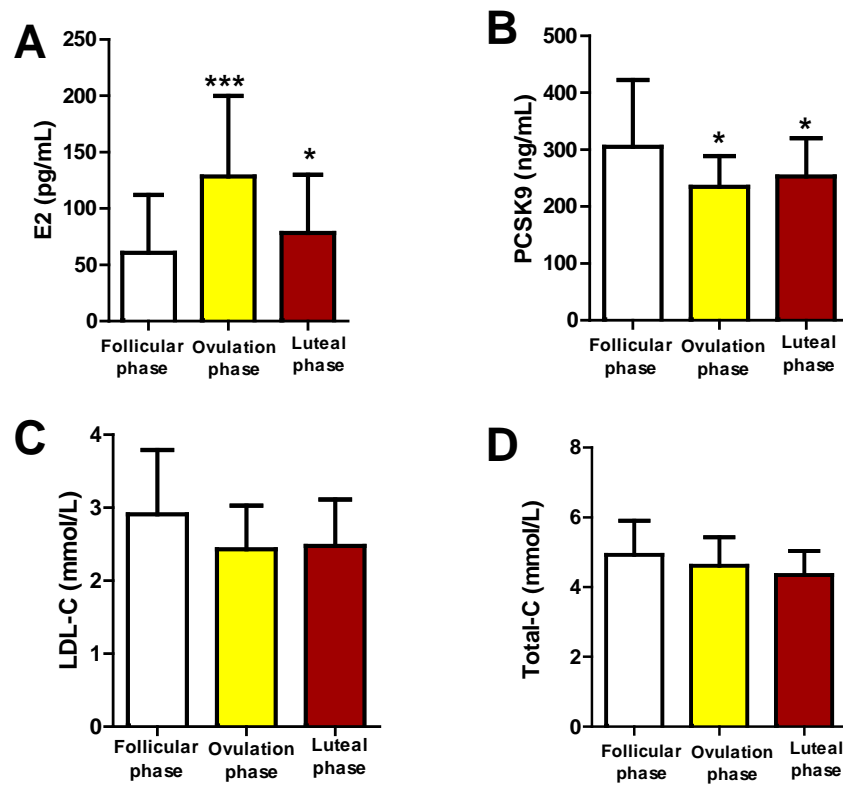


Fig 7: Changes in A) E2, B) PCSK9, C) LDL-C and D) Total-C during menstruation cycle

4.2 Paper II

Testosterone reduces PCSK9 in healthy males while cholesterol and bile acid syntheses are unaltered

Paper 1 demonstrates that plasma PCSK9 levels increase with age in females but not in males (102). The reduction in endogenous estrogen levels with age in females may partly explain this. However, on the other hand, studies have shown that cholesterol and bile acid synthesis are higher in males than in females (89, 98-100). Whether testosterone has any effects on these parameters is unclear. We tested the hypothesis that testosterone will induce bile acid and cholesterol syntheses and PCSK9 levels in normal males.

Effects of testosterone on cholesterol and bile acid synthesis

Despite marked increases of serum testosterone, we did not observe any changes in serum lathosterol, the marker of cholesterol synthesis, or C4c, an indicator of bile acid synthesis, indicating that testosterone was without effect on cholesterol synthesis or bile acid production. Serum total and individual bile acids were unchanged after 4 days of treatment but tended to increase on day 14 following the highest dose given, while there was no consistent pattern regarding conjugated or unconjugated cholic, chenodeoxycholic, deoxycholic, ursodeoxycholic or lithocholic acids. Also, the level of FGF19, reflecting bile acid interaction with intestinal FXR, was not altered.

Testosterone and serum PCSK9 and lipoproteins

Four days after injection, circulating PCSK9 levels were significantly reduced. Testosterone did not alter plasma total cholesterol or triglycerides. A trend for increased total triglyceride levels was observed 2 weeks after injection, however.

Additionally, there were no significant changes observed in VLDL-C, LDL-C or HDL-C levels on day 4. However, VLDL-C levels were increased on day 14 whereas LDL-C and HDL-C levels were decreased.

4.3 Paper III

Post-transcriptional regulation of LDLRs by PCSK9 and/or by IDOL

In papers 1 and 2, we demonstrated that hormones such as estrogen and testosterone could regulate PCSK9. In paper 3, we hypothesized that LDLRs in the adrenals and the liver might be subject to regulation by PCSK9 or IDOL upon ACTH treatment.

Post-transcriptional regulation of adrenal LDLR numbers by IDOL in response to ACTH

ACTH treatment is well known to increase LDLR mRNA levels in the adrenal gland. We hypothesized that posttranscriptional regulation of LDLRs may also occur through lowering of PCSK9 and/or IDOL in this situation. PCSK9 mRNA and protein were both undetectable in the adrenal gland. In contrast, adrenal IDOL was detected and shown to be suppressed during such treatment, both at mRNA and protein levels, indicating the involvement of IDOL in the regulation of LDLRs in the adrenal gland. These effects were more pronounced in rats than in WT mice, but were not observed in PCSK9^{-/-} mice PCSK9.

Post-transcriptional regulation of hepatic LDLRs by PCSK9 following ACTH

In rat experiments, it was shown that the downregulation of hepatic LDLRs following ACTH treatment was accompanied by increased levels of PCSK9 protein, but not its mRNA, levels. In contrast, hepatic IDOL expression was unchanged.

To study this further, PCSK9^{-/-} and WT mice were treated with and without ACTH. Similar to what was seen in rats, ACTH treatment increased serum total cholesterol and LDL-C and HDL-C in WT mice, but this was not seen in PCSK9^{-/-} mice. While LDLR mRNA was unchanged during treatment, its protein expression was reduced 50% in WT mice, there was no difference in either in PCSK9^{-/-} mice during ACTH exposure. In WT mice, PCSK9 protein was increased 2-fold by ACTH treatment, whereas both mRNA and protein levels were undetectable in PCSK9^{-/-} mice.

In addition, when we assayed PCSK9 protein in livers from adrenalectomized (ADX) rats treated with ACTH and their untreated controls, this response was abolished, indicating an adrenal gland dependent hepatic response of ACTH.

4.4 Paper IV

CDCA increases plasma LDL-C by reducing hepatic LDL-C clearance but concomitantly reduces plasma PCSK9

Increased plasma LDL-C levels have been observed during CDCA treatment in humans (101, 102). We here explored possible mechanisms behind this effect.

Three weeks of treatment with CDCA was associated with a small but significant increase in total plasma cholesterol (7%, $p < 0.05$) and LDL-C (10%, $p < 0.05$) (Fig 8A). Turnover studies utilizing autologous ^{125}I -LDL showed that, although the total amount of apoB containing lipoproteins was unchanged, the FCR of apoB in LDL was significantly reduced (Fig 8B). The onset of the FCR reduction was seen already within one day of CDCA treatment (Fig 8C).

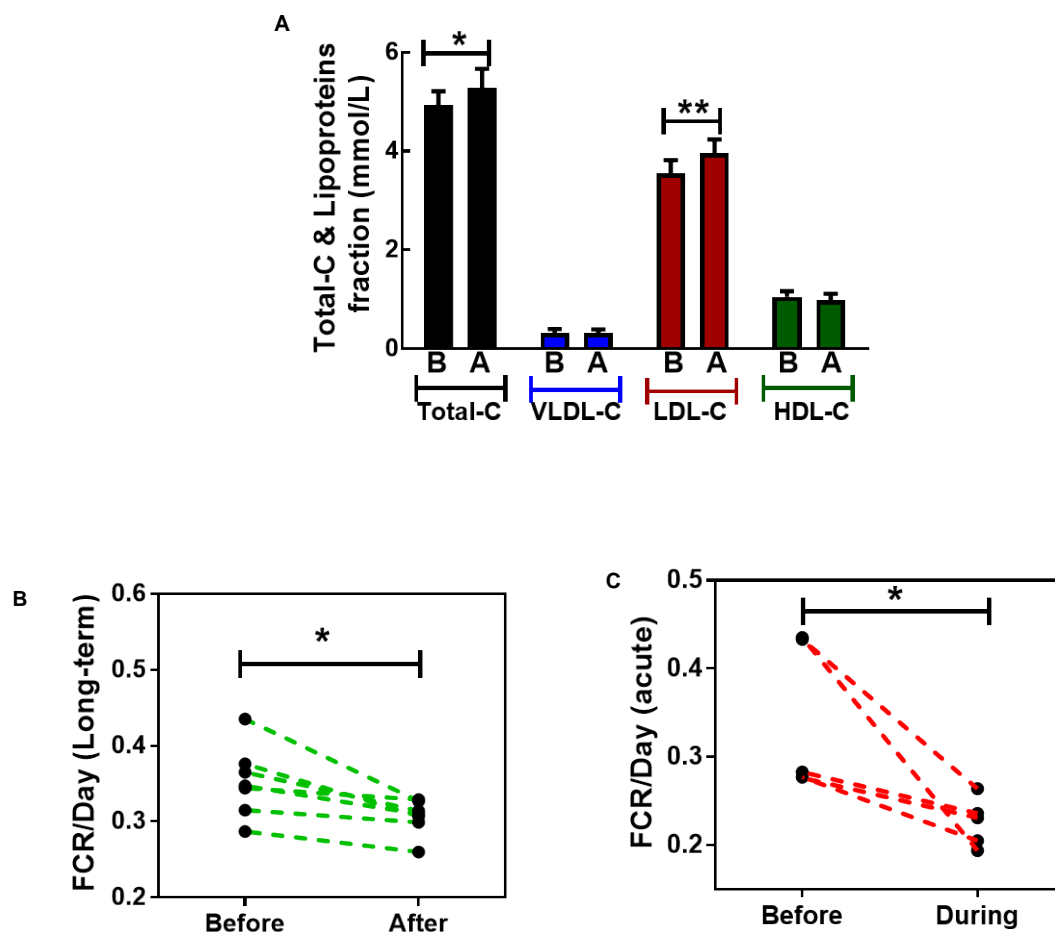


Fig 8: Changes in lipoproteins (A) and FCR, calculated from plasma (B) during 3 weeks of CDCA treatment. Acute responses of CDCA on FCR (C).

To determine if the hepatic LDL receptor binding activity was altered upon CDCA treatment, the heparin-sensitive binding of ¹²⁵I-LDL to liver homogenates was determined from liver biopsies. CDCA treatment was associated with a 20% lower ¹²⁵I-LDL binding ($P=0.09$), indicating a reduced number of LDLRs number in liver (Fig 9A). An important finding was that CDCA treatment significantly reduced circulating PCSK9 in these subjects (Fig 9B).

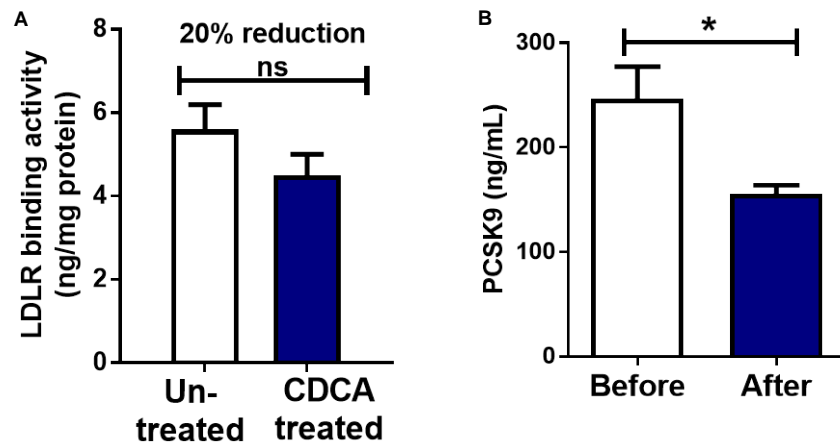


Fig 9: LDLR binding activity decreases 20% (A) together with the reduction of circulating PCSK9 (B).

5. DISCUSSION AND FUTURE PROSPECTIVE

Studies from the last two decades have established that PCSK9 is an important regulator of cholesterol metabolism and a highly promising drug target for reducing hypercholesterolemia. Recently FDA approved PCSK9 inhibitors for the treatment of hyperlipidemia. Understanding how the expression of PCSK9 may be modulated is therefore of major importance, both from physiological and pharmacological aspects. In this thesis, findings of how alterations in estrogen and testosterone levels, induction of adrenal corticosteroid production and treatment with CDCA modulate PCSK9 are reported.

5.1 Estrogen and PCSK9

Estrogen has several physiological functions. Studies have shown that increased endogenous estrogen levels are associated with decreased plasma cholesterol concomitantly with reduced PCSK9 levels in humans. However, in a population study, subjects on estrogen therapy did not show any notable changes in PCSK9 levels (63) which may indicate different effects of endogenous and exogenous estrogen on lipid metabolism as previously noticed (103). In addition, estrogen treatment has been reported to reduce hepatic PCSK9 and thereby plasma LDL-C in rats (6).

PCSK9 correlates negatively with the estrogen level in healthy women and increases markedly after menopause, suggesting an influence of endogenous estrogen levels on PCSK9 levels in females. The relation of LDL-C and PCSK9 to endogenous estrogen levels led us to hypothesize that the changes in endogenous estrogen levels during the menstruation cycle could influence PCSK9 and thus LDL-C in females. We here demonstrate in paper 1 that the estrous cycle strongly relates to PCSK9 levels in menstruating women. The changes in estrogen levels during menstruation cycles are likely to influence PCSK9 and thereby LDL-C levels as reported in normal women. However, a Chinese population study did not demonstrate any effects on PCSK9 levels during the menstruation cycle. The authors also reported that estrogen treatment at a physiological dose failed to reduce PCSK9 in human hepatocytes. In contrast, it was shown that the up-regulation of LDLRs by estrogen in human hepatocarcinoma HuH7 cells is dependent on PCSK9 (104). Our study indicates that changes in endogenous estrogen levels during menstruation or menopause could influence PCSK9 in females.

To further advance knowledge, other situations with varying estrogen levels in humans should be explored. Tentative future studies could include characterization of lipoprotein and PCSK9 levels during normal pregnancy or after sex reassignment surgery.

5.2 Testosterone and PCSK9

Little information is available on the effects of testosterone on PCSK9. It has been reported that testosterone levels in healthy males do not correlate with PCSK9 or with LDL-C (86). Moreover, testosterone replacement therapy (TRT) in hypogonadal men was unable to alter LDL-C and PCSK9 levels (86). The effects of testosterone treatment at high doses on PCSK9 in healthy males had not been studied, however. In paper 2, we showed that pharmacological doses of testosterone reduce circulating PCSK9 in healthy males. Similar results were found in pigs where hepatic and circulating PCSK9 expression was induced in pigs with castration-induced testosterone deficiency that was restored by TRT (105). Interestingly, we observed that the changes in PCSK9 levels during treatment did not correspond to the plasma LDL-C in these subjects. It is somewhat difficult to explain why the reduction of PCSK9 following testosterone is not associated with any reduction of LDL cholesterol. There are however situations where changes in serum LDL cholesterol and PCSK9 do not show the expected covariation, as we found in paper 4. Additionally, an increase in VLDL-C together with suppressed plasma LDL-C and HDL-C levels were observed 2 weeks after treatment. Such late changes in lipoproteins following testosterone treatment have indeed been reported previously (84, 106). Increased VLDL production and inhibition of hepatic lipase activity (106, 107) may be the possible explanations behind this accumulation of VLDL remnants and reduction in LDL-C and HDL-C levels.

Further animal studies would be of clear interest in order to understand in more detail the underlying mechanism.

5.3 Testosterone and gender dependent cholesterol and bile acid synthesis

Studies have shown that in human, bile acid and cholesterol production (89) and the plasma bile acid level (100) are higher in males than in females. Studies in castrated male mice have indicated that androsterone, the metabolically active product of testosterone, may be a Farnesoid X Receptor (FXR) ligand inducing expression of the FXR target gene small heterodimer partner (SHP) and thus could suppress bile acid synthesis by inhibiting CYP7A1(108). We, therefore, hypothesized that testosterone has a role in these gender-related differences.

However, it is clear from this study that testosterone has no major effects on cholesterol and bile acid synthesis in humans. Other mechanisms for this important gender difference must,

therefore, be sought. The fact that there are major species differences regarding the influence of sex on cholesterol metabolism makes it difficult to explore this question in animal models.

5.4 ACTH and PCSK9

Effects of ACTH on cholesterol metabolism have been reported previously. It was clear from a previous study that during prolonged ACTH stress, plasma cholesterol was increased in rats and mice through suppression of hepatic LDLR protein levels. In addition, this effect was absent in ADX rats, indicating an adrenal gland dependent effect of ACTH on cholesterol metabolism (3). However, LDLR mRNA levels were unaltered during ACTH exposure (3), suggesting possible post-transcriptional regulation of LDLRs by ACTH, mediated by the adrenal glands. We demonstrated in paper 3 that PCSK9 post-transcriptionally reduces hepatic LDLR numbers, thereby increasing plasma cholesterol. Additionally, we found that PCSK9 expression in liver was unaffected in ADX rats receiving ACTH, further demonstrating that the adrenal glands are critically involved in mediating the effects of ACTH on the liver (3). We speculate that the adrenal glands signal to other tissues including the liver by an unknown "factor X" to decrease lipoprotein clearance thereby elevating the cholesterol level in the blood to secure the supply of cholesterol in adrenals for corticosteroid production.

Since the protein but not the mRNA levels of PCSK9 were increased, this indicates that there is possible posttranscriptional regulation of PCSK9 itself (109). An unchanged level of circulating PCSK9 during ACTH treatment strengthens the concept that PCSK9 itself may be regulated post-transcriptionally upon ACTH treatment. This also highlights a possible involvement of intracellular variation of hepatic PCSK9, which may be important for the expression of the LDLR, as reported in other studies (110). However, in contrast to liver, the expression of PCSK9 in adrenal glands is negligible, indicating the involvement of other substitutional mechanisms for regulating LDLR in the adrenal glands.

In the future, we would like to identify "factor X" by utilizing cross circulation experiments and with proteomic, lipidomic and metabolomics screening procedures. The intracellular location of increased PCSK9 molecules in the hepatocyte following ACTH treatment and their morphological relation to the intracellular LDLR pathway would be another important aspect to consider.

5.5 ACTH and IDOL

IDOL, the recently identified regulator of cholesterol metabolism, has been reported to regulate LDLR post-transcriptionally. However, we have not noticed any obvious effects of ACTH on hepatic IDOL. However, IDOL seems to regulate LDLR in peripheral tissues, as we found in

adrenal glands. IDOL mRNA and protein expression are downregulated during ACTH exposure, which may partly explain the induced LDLR number in adrenal glands during prolonged stress. Future studies on IDOL^{-/-} mice or rats may also be of interest.

5.6 CDCA, lipoproteins and PCSK9

In paper 4, we studied why CDCA, a natural FXR agonist (111, 112), causes a moderate increase in plasma LDL-C in humans. We found that LDLRs are downregulated following CDCA treatment, thereby diminishing the clearance of LDL particles. However, studies have shown a moderate 10% induction of plasma LDL-C (101, 102). Our results also confirm that LDL-C increases about 6% just after initiation of treatment and about 13% after 3 weeks of treatment.

We found that plasma PCSK9 is significantly reduced following CDCA treatment, which could be expected as the transcription of PCSK9 is known to be regulated by SREBP2. Thus, CDCA has been shown to suppress PCSK9 mRNA and protein expression in human hepatocytes (113, 114). Additionally, CDCA reduces LDL production possibly by reducing VLDL production (115, 116). The reductions of PCSK9 levels and LDL production might, therefore, counterbalance the reduced clearance of plasma LDL-C. Additionally, we found that CDCA lowers Lp(a).

Limited information is available so far about the effects of FXR agonists on lipoproteins or apolipoproteins (117). Moreover, it is important to consider that many early trials have reported increases in LDL-C in response to synthetic FXR agonists (118, 119). In the FLINT study, plasma LDL-C increased markedly during treatment with obeticholic acid, a bile acid analog and a strong FXR agonist (120). Studies on healthy subjects have also shown that obeticholic acid treatment resulted in larger LDL particles in the plasma (121).

Therefore, it will be important to perform more detailed studies on lipoprotein metabolism following such therapy and also to understand further the influences of various synthetic FXR agonists regarding their effects on PCSK9, apoCIII and Lp(a) as seen in response to CDCA treatment in our study.

5.7 Relevance of PCSK9 regulation

In summary, this work has advanced the knowledge about the regulators of PCSK9 that modulate circulating and/or intracellular PCSK9, and thereby cholesterol metabolism. Both male and female sex hormones were found to decrease circulating PCSK9, indicating that this LDLR-modulator is under the hormonal control. In addition to hormones, CDCA, a primary bile acid, and natural FXR agonist, reduces circulating PCSK9 levels in humans. Furthermore,

this work indicates that intracellular PCSK9 post-transcriptionally regulates hepatic LDLRs and induces plasma cholesterol during prolonged stress induced by ACTH. However, these effects of PCSK9 are adrenal gland dependent, indicating that the adrenal glands signal to liver to decrease lipoprotein clearance thereby elevating the cholesterol level in the blood to secure the supply of cholesterol in adrenals for corticosteroid production. It would be interesting to investigate this signaling factor that controls cholesterol metabolism during stress.

The advancement in the knowledge about these PCSK9 regulators should eventually be advantageous for the treatment and prevention of human diseases such as atherosclerosis and dyslipidemia.

6. CONCLUSIONS

The following conclusions can be drawn from the above studies.

- 1) Circulating PCSK9 increases with age in females but not in males. PCSK9 is higher in post-menopausal females than in pre-menopausal females or in males. Physiological changes in estrogen levels during menstruation influence circulating PCSK9 levels and the level of plasma LDL-C.
- 2) Testosterone at pharmacological doses reduces circulating PCSK9 in healthy males. However, the reduction of PCSK9 following this treatment does not alter LDL-C levels. In addition, known human gender differences in bile acid and cholesterol synthesis are not likely explained by testosterone.
- 3) ACTH stimulates LDLR expression in the adrenal where suppressed IDOL levels may contribute. Following ACTH, PCSK9 post-transcriptionally suppresses hepatic LDLRs during prolonged, thereby increasing plasma cholesterol. These effects are adrenal gland dependent, and suggest the presence of a mechanism for shunting lipoprotein cholesterol to the adrenals in situations of prolonged stress.
- 4) The increase in plasma cholesterol during CDCA treatment is due to reduced plasma clearance of LDL-C via hepatic LDLRs. Concomitantly, CDCA reduces circulating PCSK9 which may counterbalance its effect on hepatic LDLRs and on plasma LDL-C. CDCA also down-regulates Lp(a) and ApoCIII in humans.

7. ACKNOWLEDGEMENTS

I would like to convey my sincere gratitude to all the people who have supported and motivated me in the journey of making this thesis:

My main supervisor Professor Mats Rudling, for accepting me as your Ph.D. student, for your valuable support and sharing of your scientific knowledge. Your dedication toward research always encouraged me.

My supervisor Professor Bo Angelin, for your invaluable support, encouragement and motivation. Your extensive knowledge and great enthusiasm for science are a huge inspiration for me.

My supervisor and Ex-colleague Dr. Lena Beckman, for introducing me to this Lab and for your nice and helpful suggestions.

Dr. Camilla Pramfalk, my mentor, for sharing your knowledge and for your helpful advice.

The head of the Department of Medicine at Huddinge, Professor Jan Bolinder and others working in the department for providing a good scientific environment.

Cecilia Gälman, Mats Eriksson, Lena Beckman, Amit Laskar, Nina Gårevik and Lena Ekström for sharing co-authorship and precious contributions to the work.

The biomedical technicians Ingela Arvidsson and Lisbet Benthin for sharing your valuable skills and expert knowledge in laboratory techniques.

The secretary Lena Emtestam, for your extensive help and guide regarding administrative matters.

Former and present colleagues at Metabolab, Novum and ICMC: Amani Al-Khaifi, Amit Laskar, Beatrice Sjöberg, Christina Savva, Daniela Strodthoff, Eva Steninger, Hong Jiao, Johanna Apro, Jonas Brinck, Marcela González, Marion Korach-Andre, Peter Benedek, Sara Straniero, Viveca Åberg, Ylva Bonde and other nurses at level 8, for the fun and interesting conversations about science and non-scientific matters.

My Ex-supervisors Dr. Xi-Ming Yuan and Dr. Wei Li, for introducing me in the area of scientific research and their support.

All the teachers of my school and college life, for their love and encouragement.

All my friends here in Sweden (Specially Shanta and Amitha) and in India (from Ranaghat, Kolkata and GCTS) for sharing a tight bond of friendship and for giving me great laugh and happiness.

My family (The biggest part of my heart):

My father (বাবা) Dayal Krishna Ghosh and my mother (মা) Ila Ghosh, for their blessings, extensive support, sacrifice and limitless love. I love both of you so much!

My sister Paramita Ghosh, for being with me in my happiness and sorrow and her husband Debraj De, the new extension of our family.

My father-in-law Ashutosh Laskar and mother-in-law Dipali Laskar, for accepting me into their family. My sister-in-law Minakshi Laskar, her husband Ranajoy Laskar, their son Anshuman Laskar and brother-in-law Sumit Laskar, for their generosity and love.

My Husband, Amit Laskar. I have no words to explain your love and extensive support. Your love, support, and understanding are the strength of my life since we are together. I feel myself luckiest because you are with me. Thank you for everything. Love you!

My Daughter, Aryana Ghosh Laskar who has completed me as a woman. The word "Mamma" in your sweet voice is the greatest gift of GOD in my life. I love you, my sweetheart!

The work was supported by the Swedish Research Council, the Stockholm City Council (ALF), the Swedish Heart-Lung Foundation, the Fondation Leducq, the NovoNordisk Foundation, the Knut and Alice Wallenberg Foundation and the Cardiovascular Program, Karolinska Institute/Stockholm City Council.

8. REFERENCES

1. Williams KJ, Tabas I. Atherosclerosis--an inflammatory disease. *The New England journal of medicine* 1999;340:1928; author reply 1929.
2. Wood D, De Backer G, Faergeman O, Graham I, Mancina G, Pyorala K. Prevention of coronary heart disease in clinical practice: recommendations of the Second Joint Task Force of European and other Societies on Coronary Prevention. *Atherosclerosis* 1998;140:199-270.
3. Galman C, Angelin B, Rudling M. Prolonged stimulation of the adrenals by corticotropin suppresses hepatic low-density lipoprotein and high-density lipoprotein receptors and increases plasma cholesterol. *Endocrinology* 2002;143:1809-1816.
4. Ness GC, Pendelton LC, Zhao Z. Thyroid hormone rapidly increases cholesterol 7 alpha-hydroxylase mRNA levels in hypophysectomized rats. *Biochimica et Biophysica Acta* 1994;1214:229-233.
5. Rudling M, Parini P, Angelin B. Effects of growth hormone on hepatic cholesterol metabolism. Lessons from studies in rats and humans. *Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society* 1999;9 Suppl A:1-7.
6. Persson L, Galman C, Angelin B, Rudling M. Importance of proprotein convertase subtilisin/kexin type 9 in the hormonal and dietary regulation of rat liver low-density lipoprotein receptors. *Endocrinology* 2009;150:1140-1146.
7. Persson L, Henriksson P, Westerlund E, Hovatta O, Angelin B, Rudling M. Endogenous estrogens lower plasma PCSK9 and LDL cholesterol but not Lp(a) or bile acid synthesis in women. *Arterioscler Thromb Vasc Biol* 2012;32:810-814.
8. Rudling M, Angelin B. Stimulation of rat hepatic low density lipoprotein receptors by glucagon. Evidence of a novel regulatory mechanism in vivo. *J Clin Invest* 1993;91:2796-2805.
9. Blackett PR, Sanghera DK. Genetic determinants of cardiometabolic risk: a proposed model for phenotype association and interaction. *Journal of clinical lipidology* 2013;7:65-81.
10. Dietschy JM, Turley SD, Spady DK. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *Journal of Lipid Research* 1993;34:1637-1659.
11. Siperstein MD, Fagan VM. Studies on the feed-back regulation of cholesterol synthesis. *Advances in enzyme regulation* 1964;2:249-264.
12. Edwards PA, Muroya H, Gould RG. In vivo demonstration of the circadian rhythm of cholesterol biosynthesis in the liver and intestine of the rat. *Journal of Lipid Research* 1972;13:396-401.
13. Gylling H, Strandberg T, Tilvis R, Miettinen TA. Regulation of serum cholesterol level in middle-aged and elderly men. Relation of cholesterol absorption and synthesis to lipoprotein metabolism. *Arteriosclerosis and thrombosis : a journal of vascular biology* 1994;14:694-700.

14. Sane AT, Sinnett D, Delvin E, Bendayan M, Marcil V, Menard D, Beaulieu JF, et al. Localization and role of NPC1L1 in cholesterol absorption in human intestine. *Journal of Lipid Research* 2006;47:2112-2120.
15. Chang TY, Chang CC, Cheng D. Acyl-coenzyme A:cholesterol acyltransferase. *Annual review of biochemistry* 1997;66:613-638.
16. Berge KE, Tian H, Graf GA, Yu L, Grishin NV, Schultz J, Kwiterovich P, et al. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science* 2000;290:1771-1775.
17. Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome research* 2001;11:1156-1166.
18. Graf GA, Yu L, Li WP, Gerard R, Tuma PL, Cohen JC, Hobbs HH. ABCG5 and ABCG8 are obligate heterodimers for protein trafficking and biliary cholesterol excretion. *The Journal of biological chemistry* 2003;278:48275-48282.
19. Abumrad NA, Davidson NO. Role of the gut in lipid homeostasis. *Physiological reviews* 2012;92:1061-1085.
20. Pan X, Hussain MM. Gut triglyceride production. *Biochimica et Biophysica Acta* 2012;1821:727-735.
21. Genest J, Choi HY. Novel Approaches for HDL-Directed Therapies. *Current atherosclerosis reports* 2017;19:55.
22. Tsompanidi EM, Brinkmeier MS, Fotiadou EH, Giakoumi SM, Kypreos KE. HDL biogenesis and functions: role of HDL quality and quantity in atherosclerosis. *Atherosclerosis* 2010;208:3-9.
23. Goldstein JL, Brown MS. Atherosclerosis: the low-density lipoprotein receptor hypothesis. *Metabolism: clinical and experimental* 1977;26:1257-1275.
24. Catapano AL, Farnier M, Foody JM, Toth PP, Tomassini JE, Brudi P, Tershakovec AM. Combination therapy in dyslipidemia: where are we now? *Atherosclerosis* 2014;237:319-335.
25. Gibbons GF, Wiggins D, Brown AM, Hebbachi AM. Synthesis and function of hepatic very-low-density lipoprotein. *Biochemical Society Transactions* 2004;32:59-64.
26. Twisk J, Gillian-Daniel DL, Tebon A, Wang L, Barrett PH, Attie AD. The role of the LDL receptor in apolipoprotein B secretion. *The Journal of clinical investigation* 2000;105:521-532.
27. Masuda D, Yamashita S. Postprandial Hyperlipidemia and Remnant Lipoproteins. *Journal of atherosclerosis and thrombosis* 2017;24:95-109.
28. Dieplinger H, Utermann G. The seventh myth of lipoprotein(a): where and how is it assembled? *Current Opinion in Lipidology* 1999;10:275-283.
29. Kronenberg F, Utermann G. Lipoprotein(a): resurrected by genetics. *Journal of internal medicine* 2013;273:6-30.
30. Koschinsky ML, Marcovina SM. Structure-function relationships in apolipoprotein(a): insights into lipoprotein(a) assembly and pathogenicity. *Current Opinion in Lipidology* 2004;15:167-174.

31. Yeang C, Witztum JL, Tsimikas S. 'LDL-C' = LDL-C + Lp(a)-C: implications of achieved ultra-low LDL-C levels in the proprotein convertase subtilisin/kexin type 9 era of potent LDL-C lowering. *Current Opinion in Lipidology* 2015;26:169-178.
32. Galman C, Arvidsson I, Angelin B, Rudling M. Monitoring hepatic cholesterol 7 α -hydroxylase activity by assay of the stable bile acid intermediate 7 α -hydroxy-4-cholesten-3-one in peripheral blood. *Journal of Lipid Research* 2003;44:859-866.
33. Modica S, Gadaleta RM, Moschetta A. Deciphering the nuclear bile acid receptor FXR paradigm. *Nuclear receptor signaling* 2010;8:e005.
34. Angelin B, Bjorkhem I, Einarsson K, Ewerth S. Hepatic uptake of bile acids in man. Fasting and postprandial concentrations of individual bile acids in portal venous and systemic blood serum. *The Journal of clinical investigation* 1982;70:724-731.
35. Ridlon JM, Kang DJ, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. *Journal of Lipid Research* 2006;47:241-259.
36. Alrefai WA, Gill RK. Bile acid transporters: structure, function, regulation and pathophysiological implications. *Pharmaceutical research* 2007;24:1803-1823.
37. Kullak-Ublick GA, Stieger B, Meier PJ. Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology* 2004;126:322-342.
38. Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, Mangelsdorf DJ. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. *Molecular cell* 2000;6:507-515.
39. Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, et al. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Molecular cell* 2000;6:517-526.
40. Holt JA, Luo G, Billin AN, Bisi J, McNeill YY, Kozarsky KF, Donahee M, et al. Definition of a novel growth factor-dependent signal cascade for the suppression of bile acid biosynthesis. *Genes & development* 2003;17:1581-1591.
41. Katafuchi T, Esterhazy D, Lemoff A, Ding X, Sondhi V, Klier SA, Mirzaei H, et al. Detection of FGF15 in plasma by stable isotope standards and capture by anti-peptide antibodies and targeted mass spectrometry. *Cell metabolism* 2015;21:898-904.
42. Goldstein JL, Brown MS. The LDL receptor. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2009;29:431-438.
43. Rudling MJ, Reihner E, Einarsson K, Ewerth S, Angelin B. Low density lipoprotein receptor-binding activity in human tissues: quantitative importance of hepatic receptors and evidence for regulation of their expression in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 1990;87:3469-3473.
44. Innerarity TL, Mahley RW. Enhanced binding by cultured human fibroblasts of apo-E-containing lipoproteins as compared with low density lipoproteins. *Biochemistry* 1978;17:1440-1447.
45. Rudenko G, Deisenhofer J. The low-density lipoprotein receptor: ligands, debates and lore. *Current opinion in structural biology* 2003;13:683-689.
46. Goldstein JL, Anderson RG, Brown MS. Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature* 1979;279:679-685.

47. Marduel M, Carrie A, Sassolas A, Devillers M, Carreau V, Di Filippo M, Erlich D, et al. Molecular spectrum of autosomal dominant hypercholesterolemia in France. *Human mutation* 2010;31:E1811-1824.
48. Tosi I, Toledo-Leiva P, Neuwirth C, Naoumova RP, Soutar AK. Genetic defects causing familial hypercholesterolaemia: identification of deletions and duplications in the LDL-receptor gene and summary of all mutations found in patients attending the Hammersmith Hospital Lipid Clinic. *Atherosclerosis* 2007;194:102-111.
49. Smith JR, Osborne TF, Goldstein JL, Brown MS. Identification of nucleotides responsible for enhancer activity of sterol regulatory element in low density lipoprotein receptor gene. *The Journal of biological chemistry* 1990;265:2306-2310.
50. Scotti E, Hong C, Yoshinaga Y, Tu Y, Hu Y, Zelcer N, Boyadjian R, et al. Targeted disruption of the idol gene alters cellular regulation of the low-density lipoprotein receptor by sterols and liver x receptor agonists. *Molecular and Cellular Biology* 2011;31:1885-1893.
51. Abifadel M, Varret M, Rabes JP, Allard D, Ouguerram K, Devillers M, Cruaud C, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nature Genetics* 2003;34:154-156.
52. Seidah NG, Benjannet S, Wickham L, Marcinkiewicz J, Jasmin SB, Stifani S, Basak A, et al. The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. *Proceedings of the National Academy of Sciences of the United States of America* 2003;100:928-933.
53. Duff CJ, Hooper NM. PCSK9: an emerging target for treatment of hypercholesterolemia. *Expert opinion on therapeutic targets* 2011;15:157-168.
54. Bergeron N, Phan BA, Ding Y, Fong A, Krauss RM. Proprotein Convertase Subtilisin/Kexin Type 9 Inhibition: A New Therapeutic Mechanism for Reducing Cardiovascular Disease Risk. *Circulation* 2015;132:1648-1666.
55. Seidah NG. PCSK9 as a therapeutic target of dyslipidemia. *Expert opinion on therapeutic targets* 2009;13:19-28.
56. Zhang DW, Lagace TA, Garuti R, Zhao Z, McDonald M, Horton JD, Cohen JC, et al. Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. *The Journal of biological chemistry* 2007;282:18602-18612.
57. Kwon HJ, Lagace TA, McNutt MC, Horton JD, Deisenhofer J. Molecular basis for LDL receptor recognition by PCSK9. *Proceedings of the National Academy of Sciences of the United States of America* 2008;105:1820-1825.
58. Farnier M. PCSK9 inhibitors. *Current Opinion in Lipidology* 2013;24:251-258.
59. Fisher TS, Lo Surdo P, Pandit S, Mattu M, Santoro JC, Wisniewski D, Cummings RT, et al. Effects of pH and low density lipoprotein (LDL) on PCSK9-dependent LDL receptor regulation. *The Journal of biological chemistry* 2007;282:20502-20512.
60. Nassoury N, Blasiolo DA, Tebon Oler A, Benjannet S, Hamelin J, Poupon V, McPherson PS, et al. The cellular trafficking of the secretory proprotein convertase PCSK9 and its dependence on the LDLR. *Traffic* 2007;8:718-732.

61. Maxwell KN, Fisher EA, Breslow JL. Overexpression of PCSK9 accelerates the degradation of the LDLR in a post-endoplasmic reticulum compartment. *Proceedings of the National Academy of Sciences of the United States of America* 2005;102:2069-2074.
62. Cui Q, Ju X, Yang T, Zhang M, Tang W, Chen Q, Hu Y, et al. Serum PCSK9 is associated with multiple metabolic factors in a large Han Chinese population. *Atherosclerosis* 2010;213:632-636.
63. Lakoski SG, Lagace TA, Cohen JC, Horton JD, Hobbs HH. Genetic and metabolic determinants of plasma PCSK9 levels. *The Journal of clinical endocrinology and metabolism* 2009;94:2537-2543.
64. Calandra S, Tarugi P, Speedy HE, Dean AF, Bertolini S, Shoulders CC. Mechanisms and genetic determinants regulating sterol absorption, circulating LDL levels, and sterol elimination: implications for classification and disease risk. *Journal of Lipid Research* 2011;52:1885-1926.
65. Costet P, Cariou B, Lambert G, Lalanne F, Lardeux B, Jarnoux AL, Grefhorst A, et al. Hepatic PCSK9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c. *The Journal of biological chemistry* 2006;281:6211-6218.
66. Bjermo H, Iggman D, Kullberg J, Dahlman I, Johansson L, Persson L, Berglund J, et al. Effects of n-6 PUFAs compared with SFAs on liver fat, lipoproteins, and inflammation in abdominal obesity: a randomized controlled trial. *The American journal of clinical nutrition* 2012;95:1003-1012.
67. Graversen CB, Lundbye-Christensen S, Thomsen B, Christensen JH, Schmidt EB. Marine n-3 polyunsaturated fatty acids lower plasma proprotein convertase subtilisin kexin type 9 levels in pre- and postmenopausal women: A randomised study. *Vascular pharmacology* 2015.
68. Richard C, Couture P, Desroches S, Benjannet S, Seidah NG, Lichtenstein AH, Lamarche B. Effect of the Mediterranean diet with and without weight loss on surrogate markers of cholesterol homeostasis in men with the metabolic syndrome. *The British journal of nutrition* 2012;107:705-711.
69. Browning JD, Horton JD. Fasting reduces plasma proprotein convertase, subtilisin/kexin type 9 and cholesterol biosynthesis in humans. *Journal of Lipid Research* 2010;51:3359-3363.
70. Persson L, Cao G, Stahle L, Sjoberg BG, Troutt JS, Konrad RJ, Galman C, et al. Circulating proprotein convertase subtilisin kexin type 9 has a diurnal rhythm synchronous with cholesterol synthesis and is reduced by fasting in humans. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2010;30:2666-2672.
71. Cui CJ, Li S, Li JJ. PCSK9 and its modulation. *Clinica chimica acta; international journal of clinical chemistry* 2015;440:79-86.
72. Sorrentino V, Fouchier SW, Motazacker MM, Nelson JK, Defesche JC, Dallong-Thie GM, Kastelein JJ, et al. Identification of a loss-of-function inducible degrader of the low-density lipoprotein receptor variant in individuals with low circulating low-density lipoprotein. *European Heart Journal* 2013;34:1292-1297.
73. Weissglas-Volkov D, Calkin AC, Tusie-Luna T, Sinsheimer JS, Zelcer N, Riba L, Tino AM, et al. The N342S MYLIP polymorphism is associated with high total cholesterol

and increased LDL receptor degradation in humans. *The Journal of clinical investigation* 2011;121:3062-3071.

74. Zhang L, Reue K, Fong LG, Young SG, Tontonoz P. Feedback regulation of cholesterol uptake by the LXR-IDOL-LDLR axis. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2012;32:2541-2546.

75. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* 1996;383:728-731.

76. Zelcer N, Hong C, Boyadjian R, Tontonoz P. LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. *Science* 2009;325:100-104.

77. Scotti E, Calamai M, Goulbourne CN, Zhang L, Hong C, Lin RR, Choi J, et al. IDOL stimulates clathrin-independent endocytosis and multivesicular body-mediated lysosomal degradation of the low-density lipoprotein receptor. *Molecular and Cellular Biology* 2013;33:1503-1514.

78. Calkin AC, Gault BT, Zhang L, Fairall L, Hong C, Schwabe JW, Tontonoz P. FERM-dependent E3 ligase recognition is a conserved mechanism for targeted degradation of lipoprotein receptors. *Proceedings of the National Academy of Sciences of the United States of America* 2011;108:20107-20112.

79. Hong C, Duit S, Jalonon P, Out R, Scheer L, Sorrentino V, Boyadjian R, et al. The E3 ubiquitin ligase IDOL induces the degradation of the low density lipoprotein receptor family members VLDLR and ApoER2. *The Journal of biological chemistry* 2010;285:19720-19726.

80. Thomas MP, Potter BV. The structural biology of oestrogen metabolism. *J Steroid Biochem Mol Biol* 2013;137:27-49.

81. Berglund L, Carlstrom K, Stege R, Gottlieb C, Eriksson M, Angelin B, Henriksson P. Hormonal regulation of serum lipoprotein (a) levels: effects of parenteral administration of estrogen or testosterone in males. *The Journal of clinical endocrinology and metabolism* 1996;81:2633-2637.

82. Enk L, Silfverstolpe G, Crona N. Dose and duration effects of oestradiol valerate on serum apolipoproteins A1 and B. *Maturitas* 1987;9:33-39.

83. Walters KA, Handelsman DJ. Role of androgens in the ovary. *Mol Cell Endocrinol* 2018;465:36-47.

84. Anderson RA, Wallace EM, Wu FC. Effect of testosterone enanthate on serum lipoproteins in man. *Contraception* 1995;52:115-119.

85. Cai Z, Xi H, Pan Y, Jiang X, Chen L, Cai Y, Zhu K, et al. Effect of testosterone deficiency on cholesterol metabolism in pigs fed a high-fat and high-cholesterol diet. *Lipids Health Dis* 2015;14:18.

86. Ooi TC, Raymond A, Cousins M, Favreau C, Taljaard M, Gavin C, Jolly EE, et al. Relationship between testosterone, estradiol and circulating PCSK9: Cross-sectional and interventional studies in humans. *Clinica chimica acta; international journal of clinical chemistry* 2015;446:97-104.

87. Axelrod J, Reisine TD. Stress hormones: their interaction and regulation. *Science* 1984;224:452-459.

88. Aguilera G. Regulation of pituitary ACTH secretion during chronic stress. *Frontiers in Neuroendocrinology* 1994;15:321-350.

89. Galman C, Angelin B, Rudling M. Pronounced variation in bile acid synthesis in humans is related to gender, hypertriglyceridaemia and circulating levels of fibroblast growth factor 19. *Journal of Internal Medicine* 2011;270:580-588.
90. Garevik N, Rane A, Bjorkhem-Bergman L, Ekstrom L. Effects of different doses of testosterone on gonadotropins, 25-hydroxyvitamin D3, and blood lipids in healthy men. *Substance abuse and rehabilitation* 2014;5:121-127.
91. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical chemistry* 1972;18:499-502.
92. Sjoberg BG, Straniero S, Angelin B, Rudling M. Cholestyramine treatment of healthy humans rapidly induces transient hypertriglyceridemia when treatment is initiated. *American journal of physiology. Endocrinology and metabolism* 2017;313:E167-E174.
93. Ghosh Laskar M, Eriksson M, Rudling M, Angelin B. Treatment with the natural FXR agonist chenodeoxycholic acid reduces clearance of plasma LDL whilst decreasing circulating PCSK9, lipoprotein(a) and apolipoprotein C-III. *Journal of Internal Medicine* 2017;281:575-585.
94. Apro J, Beckman L, Angelin B, Rudling M. Influence of dietary sugar on cholesterol and bile acid metabolism in the rat: Marked reduction of hepatic Abcg5/8 expression following sucrose ingestion. *Biochemical and biophysical research communications* 2015;461:592-597.
95. Angelin B, Einarsson K, Liljeqvist L, Nilsell K, Heller RA. 3-hydroxy-3-methylglutaryl coenzyme A reductase in human liver microsomes: active and inactive forms and cross-reactivity with antibody against rat liver enzyme. *Journal of Lipid Research* 1984;25:1159-1166.
96. Einarsson K, Angelin B, Ewerth S, Nilsell K, Bjorkhem I. Bile acid synthesis in man: assay of hepatic microsomal cholesterol 7 alpha-hydroxylase activity by isotope dilution-mass spectrometry. *Journal of Lipid Research* 1986;27:82-88.
97. Rudling MJ, Peterson CO. A simple binding assay for the determination of low-density lipoprotein receptors in cell homogenates. *Biochimica et Biophysica Acta* 1985;833:359-365.
98. Einarsson K, Hellstrom K, Kallner M. Bile acid kinetics in relation to sex, serum lipids, body weights, and gallbladder disease in patients with various types of hyperlipoproteinemia. *The Journal of clinical investigation* 1974;54:1301-1311.
99. Einarsson K, Nilsell K, Leijd B, Angelin B. Influence of age on secretion of cholesterol and synthesis of bile acids by the liver. *The New England journal of medicine* 1985;313:277-282.
100. Frommherz L, Bub A, Hummel E, Rist MJ, Roth A, Watzl B, Kulling SE. Age-Related Changes of Plasma Bile Acid Concentrations in Healthy Adults--Results from the Cross-Sectional KarMeN Study. *PloS one* 2016;11:e0153959.
101. Albers JJ, Grundy SM, Cleary PA, Small DM, Lachin JM, Schoenfield LJ. National Cooperative Gallstone Study: the effect of chenodeoxycholic acid on lipoproteins and apolipoproteins. *Gastroenterology* 1982;82:638-646.
102. Perez-Aguilar F, Breto M, Alegre B, Berenguer J. Increase in serum total cholesterol and low-density lipoprotein cholesterol by high-dose chenodeoxycholic acid in

patients with radiolucent gallstones significantly reversed during preventive low dose after gallstone dissolution. *Digestion* 1985;31:225-233.

103. Silfverstolpe G, Gustafson A, Samsioe G, Svanborg A. Lipid metabolic studies in oophorectomized women: effects induced by two different estrogens on serum lipids and lipoproteins. *Gynecologic and obstetric investigation* 1980;11:161-169.

104. Starr AE, Lemieux V, Noad J, Moore JI, Dewpura T, Raymond A, Chretien M, et al. beta-Estradiol results in a proprotein convertase subtilisin/kexin type 9-dependent increase in low-density lipoprotein receptor levels in human hepatic HuH7 cells. *FEBS J* 2015;282:2682-2696.

105. Cai Z, Xi H, Pan Y, Jiang X, Chen L, Cai Y, Zhu K, et al. Effect of testosterone deficiency on cholesterol metabolism in pigs fed a high-fat and high-cholesterol diet. *Lipids in health and disease* 2015;14:18.

106. Herbst KL, Amory JK, Brunzell JD, Chansky HA, Bremner WJ. Testosterone administration to men increases hepatic lipase activity and decreases HDL and LDL size in 3 wk. *Am J Physiol Endocrinol Metab* 2003;284:E1112-1118.

107. Tikkanen MJ, Nikkila EA. Regulation of hepatic lipase and serum lipoproteins by sex steroids. *Am Heart J* 1987;113:562-567.

108. Wang S, Lai K, Moy FJ, Bhat A, Hartman HB, Evans MJ. The nuclear hormone receptor farnesoid X receptor (FXR) is activated by androsterone. *Endocrinology* 2006;147:4025-4033.

109. Seidah NG, Awan Z, Chretien M, Mbikay M. PCSK9: a key modulator of cardiovascular health. *Circulation Research* 2014;114:1022-1036.

110. Poirier S, Mayer G, Poupon V, McPherson PS, Desjardins R, Ly K, Asselin MC, et al. Dissection of the endogenous cellular pathways of PCSK9-induced low density lipoprotein receptor degradation: evidence for an intracellular route. *The Journal of biological chemistry* 2009;284:28856-28864.

111. Bramlett KS, Yao S, Burris TP. Correlation of farnesoid X receptor coactivator recruitment and cholesterol 7alpha-hydroxylase gene repression by bile acids. *Molecular Genetics and Metabolism* 2000;71:609-615.

112. Chiang JY, Kimmel R, Weinberger C, Stroup D. Farnesoid X receptor responds to bile acids and represses cholesterol 7alpha-hydroxylase gene (CYP7A1) transcription. *The Journal of biological chemistry* 2000;275:10918-10924.

113. Nilsson LM, Abrahamsson A, Sahlin S, Gustafsson U, Angelin B, Parini P, Einarsson C. Bile acids and lipoprotein metabolism: effects of cholestyramine and chenodeoxycholic acid on human hepatic mRNA expression. *Biochemical and Biophysical Research Communications* 2007;357:707-711.

114. Langhi C, Le May C, Kourimate S, Caron S, Staels B, Krempf M, Costet P, et al. Activation of the farnesoid X receptor represses PCSK9 expression in human hepatocytes. *FEBS Letters* 2008;582:949-955.

115. Angelin B, Einarsson K, Hellstrom K, Leijd B. Effects of cholestyramine and chenodeoxycholic acid on the metabolism of endogenous triglyceride in hyperlipoproteinemia. *Journal of Lipid Research* 1978;19:1017-1024.

116. Begemann F. Influence of chenodeoxycholic acid on the kinetics of endogenous triglyceride transport in man. *European Journal of Clinical Investigation* 1978;8:283-288.

117. Pencek R, Marmon T, Roth JD, Liberman A, Hooshmand-Rad R, Young M. Effects of Obeticholic Acid on Lipoprotein Metabolism in Healthy Volunteers. *Diabetes, obesity & metabolism* 2016.
118. Ali AH, Carey EJ, Lindor KD. Recent advances in the development of farnesoid X receptor agonists. *Annals of translational medicine* 2015;3:5.
119. Mudaliar S, Henry RR, Sanyal AJ, Morrow L, Marschall HU, Kipnes M, Adorini L, et al. Efficacy and safety of the farnesoid X receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease. *Gastroenterology* 2013;145:574-582 e571.
120. Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, Chalasani N, et al. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. *Lancet* 2015;385:956-965.
121. Pencek R, Marmon T, Roth JD, Liberman A, Hooshmand-Rad R, Young MA. Effects of obeticholic acid on lipoprotein metabolism in healthy volunteers. *Diabetes Obes Metab* 2016;18:936-940.

